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Characterisation of GlialCAM, a novel cell adhesion molecule

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How to cite

FAVRE-KONTULA, Linda. Characterisation of GlialCAM, a novel cell adhesion molecule. 2008. doi: 10.13097/archive-ouverte/unige:2295

This publication URL: <https://archive-ouverte.unige.ch/unige:2295>

Publication DOI: [10.13097/archive-ouverte/unige:2295](https://doi.org/10.13097/archive-ouverte/unige:2295)

UNIVERSITÉ DE GENÈVE

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MERCK SERONO INTERNATIONAL S.A.
GENEVA RESEARCH CENTER
Docteur Bruno Antonsson

Characterisation of GlialCAM, a Novel Cell Adhesion Molecule

THÈSE

présentée à la Faculté des Sciences de l'Université de Genève
pour obtenir le grade de Docteur ès sciences, mention biologie

par

Linda Favre-Kontula

de

Helsinki, Finland

Thèse N° 4018

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2008



**UNIVERSITÉ
DE GENÈVE**

FACULTÉ DES SCIENCES

***Doctorat ès sciences
mention biologie***

Thèse de *Madame Linda FAVRE*

intitulée :

**"Characterisation of GlialCAM,
a Novel Cell Adhesion Molecule"**

La Faculté des sciences, sur le préavis de Messieurs J.-C. MARTINOU professeur ordinaire et codirecteur de thèse (Département de biologie cellulaire), B. ANTONSSON, Docteur et codirecteur de thèse (Merck Serono International S.A., Geneva Research Center, Département de biochimie de protéines, Genève, Suisse) et R. SADOUL, professeur (Centre de recherche, Equipe Neurodégénérescence et plasticité, Institut des Neurosciences, Université Joseph Fourier, Grenoble, La Tronche, France), autorise l'impression de la présente thèse, sans exprimer d'opinion sur les propositions qui y sont énoncées.

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Nombre d'exemplaires à livrer par colis séparé à la Faculté : - 4 -

RESEARCH COMMUNICATIONS AND PATENTS

PUBLICATIONS

Parts of the work described in this thesis have been published in the following articles:

- GlialCAM, an Immunoglobulin-Like Cell Adhesion Molecule is Expressed in Glial Cells of the Central Nervous System
Favre-Kontula L., Rolland A., Bernasconi L., Karmirantzou M., Power C., Antonsson B. and Boschert U. .equal contribution
Glia. 2008 Apr 15;56(6):633-45.
- Detection and identification of plasma proteins that bind GlialCAM using ProteinChip arrays, SELDI-TOF MS, and nano-LC MS/MS.
Favre-Kontula L., Sattoune-Roche P, Magnenat E, Proudfoot AE, Boschert U, Xenarios I, Vilbois F, Antonsson B.
Proteomics. 2008 Jan;8(2):378-88.
- GlialCAM enhances OPC migration *in vitro*, plays a role in CNS myelination and is downregulated in human gliomas.
Favre-Kontula L., Rolland A., Alliod, C, Frossard M, Dauvillier J., Boschert U., Pouly S. and Antonsson B.
Paper in preparation

Publications that were not included in the thesis but in which I was involved during the course of my PhD thesis are included in the Annex:

- Quantitative detection of therapeutic proteins and their metabolites in serum using antibody-coupled ProteinChip Arrays and SELDI-TOF-MS.
Favre-Kontula L., Johnson Z, Steinhoff T, Frauenschuh A, Vilbois F, Proudfoot AE.
J Immunol Methods. 2006 Dec 20;317(1-2):152-62.

PATENT

This work gave rise to the following patent application:

- The use of an immunoglobulin domain-containing cell surface recognition molecule for treating diseases. **Kontula, L.**, Proudfoot A., Vilbois F., Antonsson B. International publication number: WO/2006/120160.

Acknowledgements

First and foremost I would like to thank my thesis supervisor Bruno, for his support, and for sharing his vast biochemical knowledge with me. His trust in my work gave me the confidence and motivation to explore new approaches and research directions.

I would like to thank Prof. Jean-Claude Martinou, my supervisor at the university, for his scientific advice and support and Prof. Rémy Sadoul for accepting to review my thesis and to be a member of the jury of my thesis.

I am forever grateful to Alexandre Rolland who worked closely with me on this project, we shared the good and the hard times of the study. I am also utterly grateful to Ursula Boschert for her encouragement and guidance throughout the thesis. I am sincerely thankful to Sandrine Pouly for her scientific advice and for critically reading my manuscripts, and to Chantal Alliod and Marie-José Frossard, for their help in the work involving primary oligodendrocytes. I would like to thank the members of the Protein and Cell Sciences department and especially, Jean-Philippe Gaudry and Christian Arod, two excellent scientists and sincere, helpful and kind individuals with whom I have shared the lab. I have thoroughly enjoyed our discussions and laughs during the years. I thank Laurent Chevalet for letting me do my thesis in his department.

My sincere gratitude goes to the persons, especially Christine Power, involved in initiating the project on GlialCAM. My strong gratitude goes to George Feger, Amanda Proudfoot and the members of the student comity for critical feedback and for giving me the opportunity to do my thesis in the Institute. Furthermore, I would like to thank all the members of the institute. I am very grateful for the scientific help and advice that I have received from numerous persons. It has been a very friendly and inspiring setting to do my PhD thesis.

Last but not least, my outmost gratitude goes to my family and friends. I am very grateful to my parents for giving me an upbringing rich in experiences, trust and guidance, my sister and brother for simply being excellent and my aunt for her support. I am really taken back by the amazing friends that surround me, especially the closest of the close Julia, Fatima, Anna, Laura, Magalie and Hanna. There is no better constructive boost than the good times spent together during the challenging moments of the thesis. Finally, I want to thank my husband for his affection, constant encouragements, and sense of humour, as well as for supporting me when I am insupportable.

Résumé

Les molécules d'adhérence cellulaire (CAMs) sont des protéines de la membrane plasmique qui régissent les interactions entre les cellules ou entre les cellules et la matrice extracellulaire. Les CAMs sont généralement divisées en 4 familles en raison de leurs structures et fonctions distinctes : les cadhérines, les sélectines, les intégrines et la super-famille des immunoglobulines. Beaucoup de fonctions cellulaires, telles que l'adhésion cellulaire, l'apoptose, la prolifération, la motilité ou encore la différenciation, requièrent des interactions cellulaires avec d'autres cellules ou avec la matrice extracellulaire (Smith, 2008). Ces événements sont importants car ils contribuent aux processus vitaux du développement, de la réponse immunitaire, ainsi que de l'organisation tissulaire et de son intégrité.

La super-famille des immunoglobulines (IgSF) inclut un large éventail de protéines aux fonctions variées comme les récepteurs de surface cellulaire, les molécules présentatrices d'antigènes, les molécules d'adhérence cellulaire et les récepteurs des cytokines. Ces protéines sont généralement associées au système immunitaire. Les membres de la IgSF sont, pour la plupart, des protéines de type I qui traversent la membrane plasmique une seule fois. Cependant environ 10% de ces protéines sont accrochées à la membrane plasmique par l'intermédiaire d'un glycoposphatidylinositol. La partie extracellulaire des membres de la IgSF contient un ou plusieurs domaines de type Ig. Les membres de la IgSF jouent également des rôles primordiaux dans le fonctionnement du système nerveux central (Shapiro et al., 2007). La myélinisation des axones par les oligodendrocytes en est un remarquable exemple.

Dans le but d'identifier de nouveaux membres de la IgSF avec une valeur thérapeutique putative, nous avons utilisé une approche génomique qui vise les récepteurs des facteurs de croissance endothéliaux vasculaires (VEGF) et les facteurs de croissance dérivés de plaquettes (PDGF) de type Ig. Nous avons identifié une séquence correspondant à une nouvelle protéine transmembranaire avec deux domaines de type Ig que nous avons nommé GlialCAM. L'ADNc a été cloné par assemblage d'exon à partir d'une librairie d'ADN de cerveau humain. Au début de ce projet, aucune publication décrivant cette protéine n'était disponible, depuis lors, cette protéine a été publiée sous le nom d'hepaCAM et Zig-1, de manière indépendante (Chung et al., 2005; Spiegel et al., 2006). Ces rapports démontrent que GlialCAM est

une molécule d'adhésion cellulaire exprimée dans les tissus humains du foie, par les cellules de Schwann primaires lorsque celles-ci sont stimulées par l'AMPc dibutyrique, ainsi que dans les nerfs sciatiques de rats nouveau-nés (Moh et al., 2005). Un rôle de gène suppresseur de tumeur humain lui a été également proposé, car la protéine est inhibée dans les carcinomes hépatiques et démontre des propriétés antiprolifératives *in vitro* (Chung et al., 2005).

Le but de notre étude est de déterminer le profil d'expression tissulaire et cellulaire de GlialCAM, sa fonction moléculaire et son possible rôle physiologique. De plus, nous souhaitons étudier le domaine extracellulaire de cette molécule afin de vérifier son potentiel pour une application thérapeutique. Effectivement, les domaines extracellulaires de plusieurs protéines CAM sont solubilisés par clivage protéolytique *in vivo* et ont des fonctions physiologiques (Diestel et al., 2005; Westphal et al., 1997).

Le but de la première partie de cette thèse est d'identifier le schéma d'expression de GlialCAM au niveau tissulaire et cellulaire chez l'humain et les rongeurs. Nous démontrons que la molécule est principalement exprimée dans le système nerveux central chez l'humain et la souris avec une prédominance d'expression observée dans les régions de la matière blanche, riches en cellules gliales, ainsi que dans les cellules ependymales des zones ventriculaires et du canal central de la moelle épinière. Aucune expression de GlialCAM n'a été observée dans les tissus et cellules hépatiques de souris et les niveaux détectés dans le foie humain étaient très faibles. Dans le cerveau, les oligodendrocytes (OLs) sont le type cellulaire principal exprimant la molécule. En se basant sur ces profils d'expression, nous avons donc nommé cette molécule: molécule gliale d'adhérence cellulaire (GlialCAM). L'expression de GlialCAM augmente pendant le développement du cerveau chez la souris, corrélant ainsi avec les changements de niveaux d'expression des protéines basiques de la myéline (MBP). Ceci est visible à différentes étapes de la différenciation des OLs *in vitro*. De plus, la protéine colocalise avec GAP43 dans les structures de croissance du cône des OLs. Enfin, GlialCAM pourrait avoir une fonction dans les astrocytes, car la protéine est détectée au niveau des contacts cellulaires chez les astrocytes *in vitro*.

Le but de la deuxième partie est d'identifier la fonction de GlialCAM dans les cellules gliales du système nerveux central et plus précisément dans les OLs. Ce travail démontre pour la première fois que GlialCAM est glycosylée dans le cerveau de souris et que la déglycosylation de la protéine conduit à sa rapide dégradation *in vitro*.

GliaCAM est également phosphorylée dans les astrocytes in vitro et qu'ERK2 et ASK1 phosphorylent son domaine intracellulaire in vitro. De plus, nous ne détectons pas de dimérisation cis de GliaCAM, alors que la protéine forme des interactions trans. La présence de la domaine extracellulaire soluble de GliaCAM (sGliaCAM) accroît la migration des cellules oligodendrocytes précurseurs (OPCs). Une hypothèse serait que la présence de sGliaCAM permet le détachement par compétition de l'interaction de GliaCAM entre OPC. D'ailleurs, les OPCs sans GliaCAM montrent une augmentation de la migration comparée au contrôle de type sauvage. Enfin, nous démontrons que GliaCAM est sous-régulé dans les oligodendrogliomes et les astrocytomes, très probablement par hyperméthylation de l'ADN.

Dans la dernière partie de ce travail, nous démontrons que chez la souris, la pré-administration de GliaCAM soluble réduit la réponse de la cytokine TNF α après stimulation avec LPS. Dans le but de déterminer comment GliaCAM soluble induit cette réponse, nous avons identifié des protéines plasmatiques qui s'attachent à GliaCAM après stimulation avec LPS. Pour ce faire, nous avons développé une nouvelle stratégie de détection et d'identification de partenaires protéiniques. Nous avons utilisé des ProteinChips et le SELDI-TOF MS pour détecter les interactions protéines-protéines. Nous avons suivi une méthode robuste pour isoler et identifier les protéines avec le SELDI-TOF MS, qui utilise des billes d'affinité, RP-HPLC, nano LC MS/MS et des outils de bioinformatique. En utilisant cette nouvelle méthode, nous avons pu isoler et identifier à partir du plasma de souris stimulée avec LPS, deux protéines, mannose binding lectin C (MBL-C) et properdin, qui interagissent avec GliaCAM soluble. Il s'agit de l'un des premiers rapports positif sur l'utilisation du SELDI-TOF MS dans l'identification de nouvelles interactions protéines-protéines à partir d'échantillons biologiques complexes.

Summary

Cell adhesion molecules (CAMs) are plasma membrane proteins that mediate cell-cell or cell-extracellular matrix (ECM) interactions. CAMs are generally divided into four major families based on their structure and function, namely the cadherins, the selectins, the integrins and the immunoglobulin super-family (IgSF). Many important cellular functions involve CAMs, including cell adhesion, survival or apoptosis, proliferation, motility and differentiation (Smith, 2008). These events are important for vital processes such as development, immune responses and tissue organisation and integrity.

The IgSF superfamily includes a vast amount of proteins with different functions such as cell surface receptors, antigen presenting molecules, cell adhesion molecules and certain cytokine receptors. They are most commonly associated with the immune system. IgSF members are mostly single transmembrane spanning proteins, however approximately 10 % of the members are anchored to the membrane through a glycosylphosphatidylinositol moiety. The extracellular region contains one or more Ig-like domains. IgSF members also play key roles for the proper functioning of the nervous system (Shapiro et al., 2007). For instance, oligodendrocyte (OLs) mediated axon myelination is a remarkable example of coordinated cell-cell adhesion.

In order to identify novel IgSF members with putative therapeutic value, we used a structure-based genome-mining approach targeting vascular endothelial growth factor receptor (VEGFR) and platelet derived growth factor receptor (PDGFR) Ig-like folds. We identified a sequence corresponding to a novel single transmembrane spanning protein with two Ig-like domains. The cDNA was cloned by exon assembly from a human brain genomic DNA library and the corresponding protein named GlialCAM. When this work was initiated, there were no reports on the protein, however since then it has been published under the name hepaCAM and Zig-1, independently (Chung et al., 2005; Spiegel et al., 2006). These reports show that GlialCAM is a cell adhesion molecule expressed in human liver and dibutyryl cAMP stimulated primary rat Schwann cells and in newborn rat sciatic nerves (Moh et al., 2005). It is a putative tumour suppressor gene, because the protein is downregulated in hepatocellular carcinoma and shows antiproliferative properties *in vitro* (Chung et al., 2005).

The aim of our study was to determine the tissue and cellular expression profile of GlialCAM, its molecular function and a possible physiological role. Furthermore, we were interested in studying whether the extracellular domain could have a potential in therapeutic applications. Extracellular domains of several CAM proteins are solubilised through proteolytic cleavage *in vivo* and have physiological functions (Diestel et al., 2005; Westphal et al., 1997).

The aim of the first part of the thesis was to identify the pattern of GlialCAM expression in humans and rodents at the tissue and cellular level. We show that the molecule is mainly expressed in the CNS in both humans and mice, with the strongest expression observed in white matter regions of the brain, and in ependymal cells of the ventricular zones and the spinal cord central canal. No expression of GlialCAM could be detected in mouse liver and the levels detected in human liver were very low. In the brain, oligodendrocytes (OLs) were identified as the major cell type expressing the molecule. Based on this expression profile, we named the molecule glial cell adhesion molecule (GlialCAM). GlialCAM expression increases during mouse brain development, correlating with changes in myelin basic protein (MBP) expression levels. It is detected at various differentiation stages of OL *in vitro*. Interestingly, the protein colocalizes with GAP43 in OL growth cone-like structures. In addition, GlialCAM might have functions in astrocytes, since the protein is detected at cell contact sites in astrocytes *in vitro*.

The aim of the second part was to identify GlialCAM function in CNS glial cells, with a focus on OLs. This work demonstrates for the first time that GlialCAM is glycosylated in mouse brain and that deglycosylation of the protein leads to rapid degradation *in vitro*, that it is phosphorylated in astrocytes *in vitro* and that ERK2 and ASK1 phosphorylates the intracellular domain *in vitro*. Furthermore, we do not detect GlialCAM cis-dimerisation, while the protein forms trans-interactions. sGlialCAM enhances OPC migration, presumably due to reduced GlialCAM mediated contact between OPCs. This is supported by the fact that GlialCAM deficient OPCs show enhanced migration compared to WT controls. Furthermore we demonstrate that GlialCAM is downregulated in oligodendrogliomas and astrocytomas, most likely through DNA hypermethylation.

In the last part of this work, we demonstrate that preadministration of sGlialCAM reduces the TNF α cytokine response after LPS stimulation in mice. In order to determine how sGlialCAM exerts this response, we identified plasma proteins that bind to it following LPS stimulation. For this purpose, we developed a novel strategy for the

detection and identification of protein binding partners. This encompassed using ProteinChips and SELDI-TOF MS to detect protein–protein interactions. Followed by a robust method for isolating and identifying proteins detected with SELDI-TOF MS, using affinity beads, RP-HPLC, nano LC MS/MS and bioinformatic tools. Using this novel method, we isolated and identified from the plasma of LPS stimulated mice, two proteins, mannose binding lectin C (MBL-C) and properdin, which interact with sGlia1CAM. This is one of the first reports of successful use of SELDI-TOF MS to identify novel protein–protein interactions from complex biological samples.

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List of abbreviations

Abbreviation	Full name
%	Percentage
[³³ P _γ]-ATP	Adenosine-5'-triphosphate-γ - ³³ P
°C	Degree Celsius (centigrade)
μ	Micro
μCi	Micro Curie
μm	Micrometer
ASK1	Apoptosis Signal-regulating Kinase 1
BCA	Bicichonic acid
bp	Base pair
BS3	Bis(sulfosuccinimidyl) suberate
BSA	Bovine serum albumin
cDNA	Complementary Deoxyribonucleic acid
CK2α	Casein kinase II subunit alpha
CNS	Central nervous system
cpm	Counts per minute
d	Days
DMEM	Dulbecco's modified eagle medium
DTT	Dithiothreitol
ECM	Extracellular matrix
EDTA	Ethylenediamine-tetraacetic acid
eGFP	Enhanced green fluorescence protein
ERK2	Extracellular signal-regulated kinase 2
FBS	Fetal bovine serum
g	Gram
<i>g</i>	Gravitational force
GlialCAM-ICD	Intracellular domain of GlialCAM
h	Hours
HBSS	Hank's buffered salt solution
HEK/EBNA	HEK293 cells stably transfected with the EBNA-1 gene
HEK293	Human embryonic kidney 293 cells
His	Histidine
i.p.	Intra-peritoneal
Ig	Immunoglobulin
JNK3	c-Jun N-terminal kinase-3
kDa	Kilo Daltons
kg	Kilograms
LC MS/MS	Liquid Chromatography with Tandem Mass Spectrometry Detection
LPS	Lipopolysaccharide
M	Molar
MALDI-TOF-MS	Matrix-assisted laser desorption/ionization-time of flight-mass spectrometry
MAPK	Mitogen activated kinases
mCi	Milli Curie
MEK2	MAPK/ERK kinase 2

mg	Milligram
MgCl ₂	Magnesium dichloride
min	Minutes
ml	Millilitre
mM	Millimolar
MnCl ₂	Manganese dichloride
Na ₃ VO ₄	Sodium vanadate
NaCl	Sodium chloride
NaF	Sodium fluoride
NaN ₃	Sodium azide
NaPO ₄	Sodium phosphate
ng	Nanograms
Ni ²⁺ -NTA	Nickel-nitrilotriacetic acid
nM	Nanomolar
N-terminal	Amino-terminal
OL	Oligodendrocyte
OPC	Oligodendrocyte precursor cell
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PDGF-AA	Platelet derived growth factor AA isoform
pg	Picograms
pH	Negative log of Hydrogen ion concentration
pI	Isoelectric point
PI3Kg	Phosphoinositide-3 kinase gamma
PLC	Phospholipase C
RP-HPLC	
rpm	Revolutions per minute
RT	Room temperature (22-25 °C)
sCD147	Extracellular domain of CD147
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SEC	Size exclusion chromatography
SELDI-TOF MS	Surface-enhanced laser desorption/ionisation time of flight mass spectrometry
sGlialCAM	Extracellular domain of GlialCAM
TNFα	Tumour necrosis factor alpha
v/v	Volume per volume
w/v	Weight per volume

1.Introduction

1.1.Cell adhesion molecules

Cell adhesion molecules (CAMs) are plasma membrane proteins that mediate cell-cell or cell-extracellular matrix (ECM) adhesion. The ECM is a complex mixture of matrix molecules, including glycoproteins, fibronectins, collagens, laminins, proteoglycans, and soluble proteins including growth factors. CAMs are generally divided into four major families based on their structure and function, namely cadherins, selectins, integrins and immunoglobulin super-family (IgSF). Due to the rough topography of the cell membrane, in order to project out from the lipid bilayer and to overcome steric hindrance for connections between cells, CAMs generally have a large extracellular domain containing several domain repeats and glycosylation sites. Mostly CAMs have a single transmembrane domain and an intracellular domain of varying length. Many important cellular functions require cell-cell or cell to matrix interactions, including cell migration, proliferation and differentiation. Furthermore, CAMs have been extensively studied due to their important and critical roles in many normal biological, but also pathological conditions, including leukocyte trafficking, haemostasis, tissue organization and integrity, embryogenesis and tumour progression (Rosales et al., 1995).

CAM interactions are by no means static, since even for cells involved in tissue integrity the bonds that connect the cells are continuously created, broken and shifted to other adjacent bonds, albeit slowly (Evans and Calderwood, 2007). Fast adhesion and release is observed by CAMs involved in the initial attachment of immune cells to the blood vasculature. In this system CAMs form cell-cell bonds at an incredibly fast rate, followed alternatively by a quick release or a rapid activation of new adhesive components to immobilize the cell and guide its migration into the surrounding tissue. CAM adhesivity can be regulated by four major mechanisms, visualised in figure 1 (Kamiguchi and Lemmon, 2000).

1) Most CAMs, including cadherin, integrins and IgSF CAMs (IgCAMs) interact directly or through adaptor proteins with the actin cytoskeleton, which concentrates the

proteins in close proximity enhancing the formation of homo- or heterophilic oligomers. This in turn regulates CAM ligand binding specificity and avidity.

2) CAM levels at the cell membrane are regulated by internalisation and recycling. Here, a good example is the regulation of integrin levels on the cell membrane of migrating cells. As the cell moves forward, integrins at the rear end of the cell are internalized and recycled to the front of the cell for new adhesive contacts. Regulation of CAM activity through CAM-associated kinases and phosphatases is likely to be a general mechanism in cell motility.

3) CAM activity is also commonly regulated by protease-mediated cleavage of the ectodomain. For instance the L1 ectodomain is cleaved on melanoma cells by metalloproteases and plasmin, leading to decreased L1 adhesivity. However, very little is known to date about the normal biological relevance of ectodomain cleavage of the L1 family members.

4) In addition, CAM concentration in the cells is regulated at the transcriptional level. This regulation mechanism is however much slower than the post-translational modifications mentioned above.

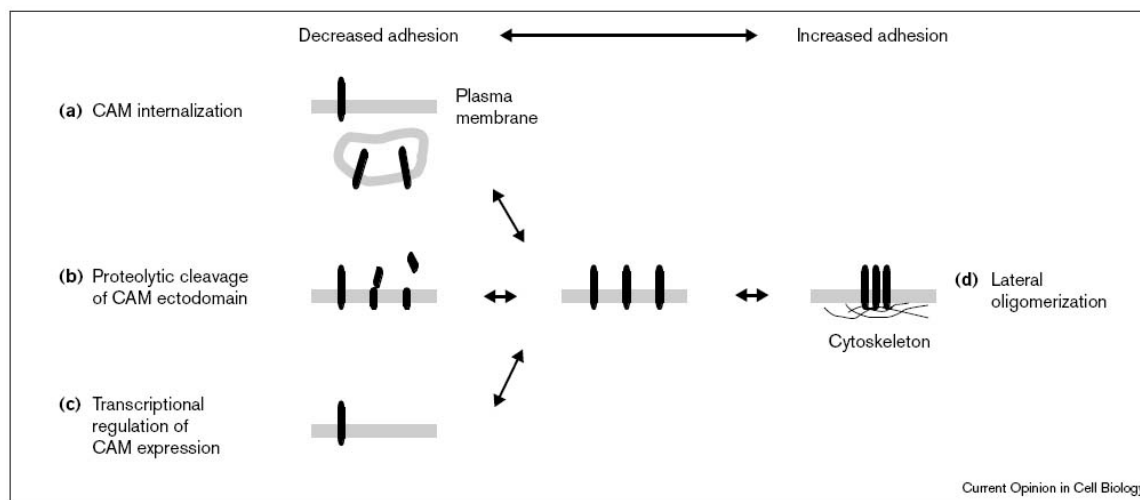


Figure 1. Four major ways of regulating CAM adhesive functions both spatially and temporally. Levels of active CAM molecules on the cell membrane and participating in adhesive mechanisms are regulated by (a) internalisation and recycling, (b) Proteolytic cleavage of the CAM ectodomain, (c) regulation of CAM expression at the transcription level and (d) spatial rearrangement through interactions with the actin cytoskeleton favouring lateral oligomerisation and hence regulating CAM activity. (From Kamiguchi et al. Curr Opin Cell Biol, 2000)

CAM adhesion events are also controlled by adhesion versus repulsion signals. Two protein pairs play a critical role in regulating this process, namely the Semaphorin-Plexin and ephrin-Eph receptor and ligand pairs (Halloran and Wolman, 2006). These systems are specifically important during development, for example for axon guidance and cell migration. Semaphorins regulate repulsion by inhibiting integrin function, while ephrins and Ephs can themselves function as cell adhesion molecules and/or initiate repulsion signals. Ephrins and Ephs controlled repulsion versus adhesion is regulated by transendocytosis, proteolytic cleavage of ephrins and specific cis- or trans- interactions between ephrins and Ephs.

1.1.1. Cadherins

Cadherins are calcium dependent cell adhesion molecules that play an important role in cell-cell contact sites such as adherens junctions (AJ) and desmosomes (Rosales et al., 1995). AJs anchor the actin-cytoskeleton to the cell contact site and confer physical strength to the tissue and are involved in morphogenesis. Desmosomes link the intermediate filaments to the intercellular junctions and are particularly important for the integrity of tissues that endure physical stress, including the epidermis and myocardium. Different cell types express specific cadherins, a feature that is important for tissue organisation, especially during embryogenesis. Loss of cadherin expression in epithelial tumours is associated with a more invasive and malignant phenotype.

The cadherin superfamily has been divided into several subfamilies and new family members are continuously being discovered. At least 80 different cadherins have been reported in a single mammalian species, belonging to the subfamilies classical cadherins, desmosomal cadherins, protocadherins, cadherin-related neuronal receptors (CNR), Fats, seven-pass transmembrane cadherins, and Ret tyrosine kinase (Figure 2) (Angst et al., 2001). A little more than twenty years ago Jacobs and co-workers described the first cadherin molecule, E-cadherin, which is part of the classical cadherin family (Hyafil et al., 1981; Peyrieras et al., 1983). The classical and desmosomal cadherins are the most studied cadherin families in the context of cell adhesion (Pokutta

and Weis, 2007), hence the focus will be placed on these. Classical cadherins are found at the AJ and are involved in the dynamics of the actin cytoskeleton through linkage with catenin proteins. Classical cadherins are further divided into type I and type II on the basis of sequence homology. Desmosomal cadherins are the transmembrane components of desmosomes and they indirectly mediate the link with the intermediate filaments. Desmosomal cadherins are sub-divided into desmogleins and desmocollins. Both classical and desmosomal cadherins have an N-terminal extracellular region containing five cadherin repeats (EC1-EC5), a transmembrane domain and an intracellular region (Shapiro et al., 2007). The cadherin repeat is composed of approximately 110 amino acids and forms a β -sandwich domain with Greek-key topology. Three calcium ions specifically bind between each cadherin repeat inducing a rigid elongated crescent shape, which is required for adhesion. Several studies show that the adhesive binding site of cadherins is localised primarily in the most N-terminal cadherin repeat (EC1). The role of the other cadherin repeats remains controversial but they are suggested to be important for the rigidity of the molecule and for lateral dimerisation (cis-dimerisation), meaning dimerisation on the same cell. It is suggested that cadherin cis-dimerisation promotes subsequent trans-dimerisation, meaning dimerisation between cadherins on opposite cells (Pokutta and Weis, 2007). Cadherins primarily form extracellular homotypic interactions and many studies show that cells expressing different subtypes of cadherins segregate when mixed. However, they can also form extracellular heterotypic interactions with other cadherins, a feature that is largely dependent on the expression levels of the different cadherins on the cells as well as the shear force that is applied to the cells. Initial cell-cell contacts are made by cadherins present on highly dynamic lamellipodia. It is suggested that the initial bonds are very short-lived and can be of heterotypic nature and it is only if the members stay in contact for a sufficient length of time, probably through homotypic interactions, so that the cell-cell contact becomes more stable and more cadherin molecules diffuse into the contacting region. However, even in established AJs the cadherin-cadherin bonds are most probably continuously formed, disrupted and reformed (Perret et al., 2004).

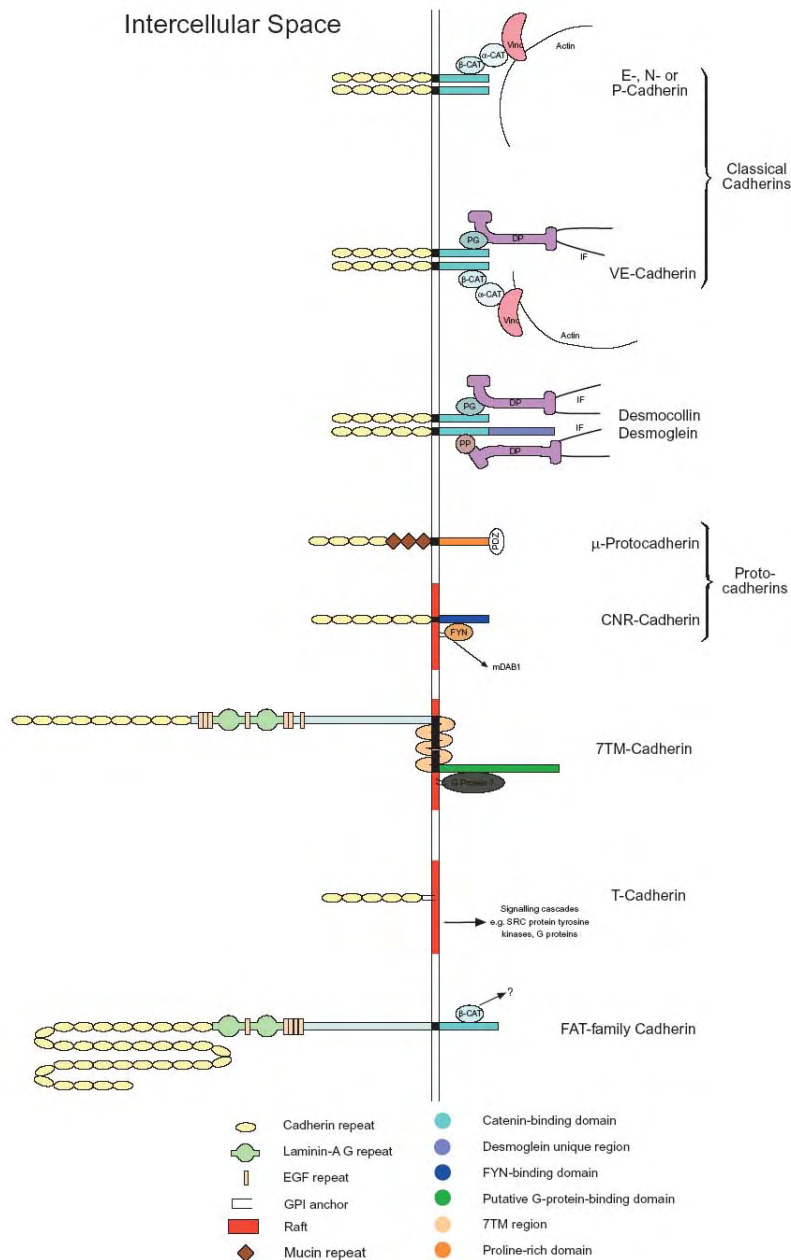


Figure 2. A schematic overview of the cadherin superfamily, where each sub-family is illustrated by one representative molecule. The abbreviations are as follows: A-CAT, α -catenin; β -CAT, β -catenin; PG, plakoglobin; PP, plakophilin; Vinc, vinculin; IF, intermediate filament; DP, desmoplakin. Desmoglein and desmocollin can both bind to PG and PP as a link to DP, but for simplicity only one interaction is depicted. Lateral dimers are shown only for classical and desmosomal cadherins because of lack of direct evidence for lateral dimerisation in the other sub-families. VE-cadherin can interact both with the IF and the actin cytoskeleton and both interactions are therefore shown on the same lateral dimer for illustrative purposes. (From Angst, B., J Cell Sciences, 2001)

The cytoplasmic domain of the classical cadherins has been extensively studied. It consists of about 150 amino acids and is the most conserved region in these proteins. In AJs it binds to β -catenin and to p120. The role of p120 remains unclear but it might control local actin regulators and cadherin endocytosis. Studies on E-cadherin have shown that E-cadherin associates with β -catenin already in the endoplasmic reticulum (ER) and the two proteins are escorted together to the plasma membrane. The interaction of E-cadherin with β -catenin is very strong and it is modulated by phosphorylation. β -catenin binds α -catenin which in return binds F-actin and it was originally believed that this forms a direct link between cadherin and the actin cytoskeleton. However, recent studies indicate that α -catenin binds to F-actin and β -catenin in a mutually exclusive manner. This has generated a new hypothesis on the sequence of events that lead to AJ formation, as shown in figure 3 (Pokutta and Weis, 2007). As previously mentioned the initial cell-cell contacts are made by cadherins on the edge of dynamic lamellipodia. The formation of the lamellipodia is driven by Arp 2/3-stimulated polymerisation of branched actin networks, a process that can be suppressed by α -catenin. When cadherins cluster through their ectodomains, a high local concentration of the cadherin- β -catenin- α -catenin complex is generated and since α -catenin readily converts between the bound and unbound forms it results in an elevated local concentration of unbound α -catenin. This leads to an α -catenin mediated suppression of Arp 2/3 activity, a cessation of lamellipodia movement and consequently a more stable cell-cell contact.

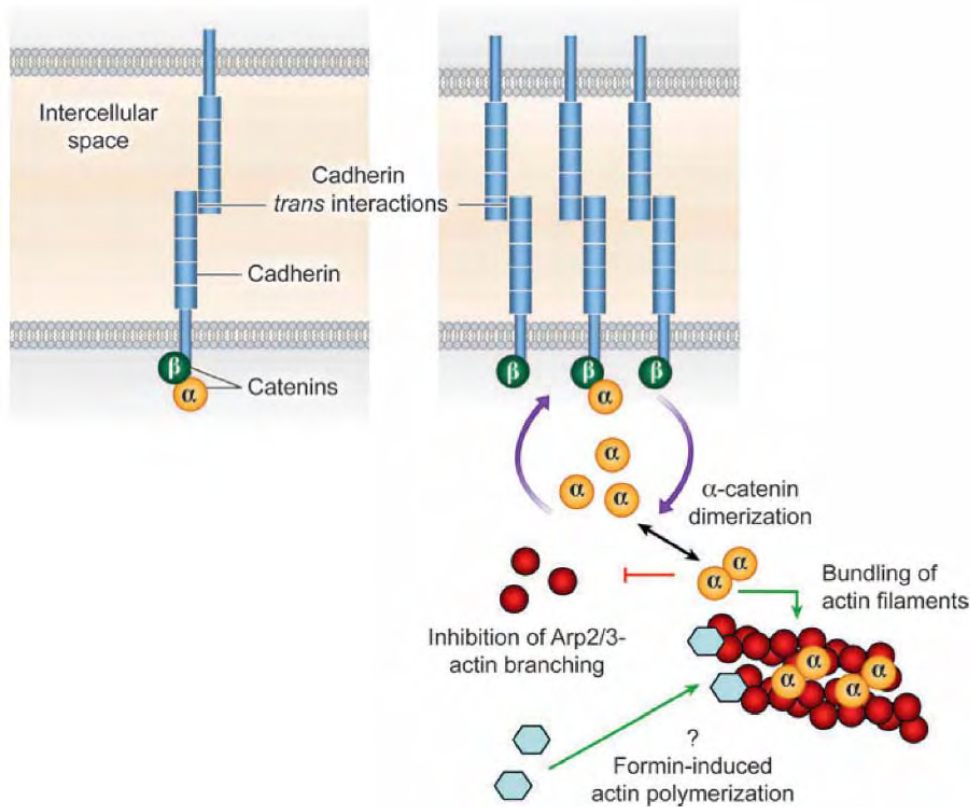


Figure 3. Proposed model of α -catenin mediated regulation of actin polymerisation at the site of newly forming adherens junctions. Initial contacts of cadherins at the tips of lamellipodia on opposing cells leads to local clustering of the E-cadherin- β -catenin- α -catenin complex. α -catenin readily exchanges between a β -catenin bound form and a dimer in the cytosol. This creates an enhanced local concentration of dimeric α -catenin which suppresses Arp2/3 mediated actin branching and the adherens junction is formed. (From Pokutta, S., Ann Rev Cell Dev Biol, 2007).

1.1.2. Selectins

The selectins are a family of calcium-dependent, type I transmembrane glycoproteins that are the principal players in leukocyte rolling, a crucial immunological process that enables leukocytes to leave the intravascular compartment and migrate into the surrounding tissue. However, these molecules also play a critical role in cancer progression and immunological diseases such as psoriasis, asthma and arthritis (Barthel et al., 2007).

In mammals, three family members with high structural conservation have been identified, P-selectin, E-selectin and S-selectin. The selectins are composed of an N-terminal lectin domain, an epidermal growth factor (EGF)-like domain, a variable number of consensus repeats (CRs), a single transmembrane domain and a short cytoplasmic domain, as depicted in figure 4 (Kneuer et al., 2006). L-selectin has two CRs, E-selectin has 6 CRs and depending on the splice variant P-selectin has 8 or 9 CRs. Soluble forms of E-selectin and P-selectin have been detected in plasma, however their biological function is not clear.

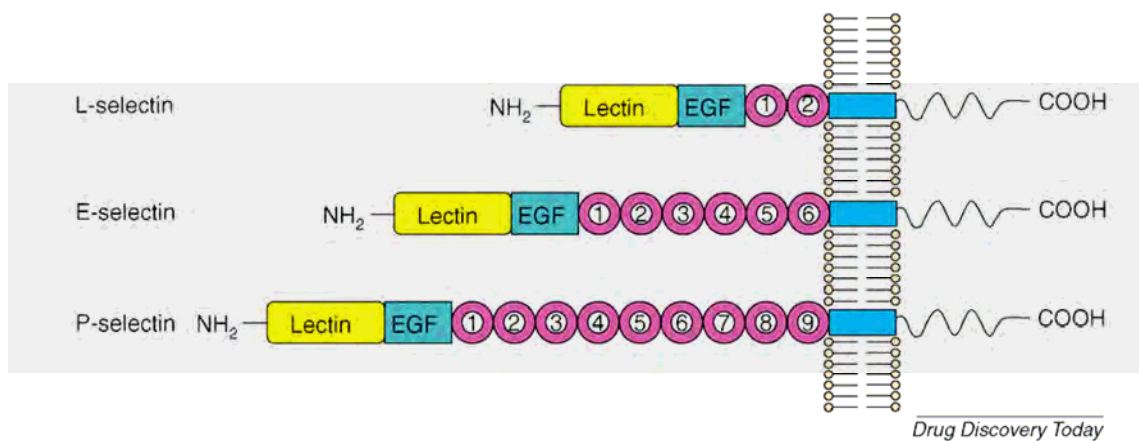


Figure 4. The selectins are composed of an N-terminal lectin domain, an epidermal growth factor (EGF)-like domain, a variable number of consensus repeats (CRs), a single transmembrane domain and a short cytoplasmic domain. The major structural difference between the three selectin types is in the number of CRs. In humans, P-selectin has 9 CRs, E-selectin has 6 CRs and L-selectin has 2 CRs. (Adapted from Kneuer, C, drug discovery today, 2006)

Selectins preferentially bind to ligands containing specific carbohydrate moieties, such as sialyl Lewis X or sialyl Lewis A which are found at the terminus of O-glycans, N-glycans and neolactosphingolipids (Sperandio, 2006). The best characterised ligand is P-selectin glycoprotein ligand-1 (PSGL-1), which was first identified as a P-selectin ligand but has a dominant role as a ligand for all three selectins. PSGL-1 is expressed in almost all leukocytes but it binds to selectins only when they harbour specific carbohydrate moieties.

Selectins are expressed exclusively in bone-marrow-derived cells and endothelial cells (Ley and Kansas, 2004). P-selectin is stored in α -granules in platelet cells and

secretory granules in resting vascular endothelial cells and can be mobilized rapidly to the cell surface after cell activation (eg, by tumor necrosis factor α (TNF α) and lipopolysaccharides (LPS). E-selectin is expressed in cytokine-stimulated (eg, by TNF α and IL-1) endothelial cells and non-inflamed skin microvessels. L-selectin is constitutively expressed on all leukocytes and its activity is regulated by metalloprotease-mediated cleavage of the extracellular domain. The main physiological function of selectins and their ligands are in the leukocyte adhesion cascade but they also have signalling functions, which are however less well understood. The leukocyte adhesion cascade involves several distinct steps, including capture (primary tethering) of the leukocytes to the vascular endothelial lining, rolling, strengthened adhesion, intravascular crawling, para- and transcellular migration and migration through the basement membrane into the adjacent tissue (Ley et al., 2007). The capture and rolling of the leukocytes is mediated by selectins (Figure 5). P-selectin and E-selectin have distinct and overlapping roles in the initial leukocyte adhesion and rolling and activation of endothelium during acute inflammation. L-selectin mediated rolling occurs mostly between free and adherent leukocytes, a process that is called secondary tethering. However, to a lesser degree it can also interact with ligands directly on the activated endothelium. Leukocyte rolling is mediated by exceptionally high on/off binding rates between selectin and their ligands and interestingly the continuous shear force exerted by the rapid blood flow is required for this process (Sperandio, 2006). Selectin binding adjusts to increasing forces by decreasing off-rates, meaning that with increasing blood flow selectin bonds lock more tightly.

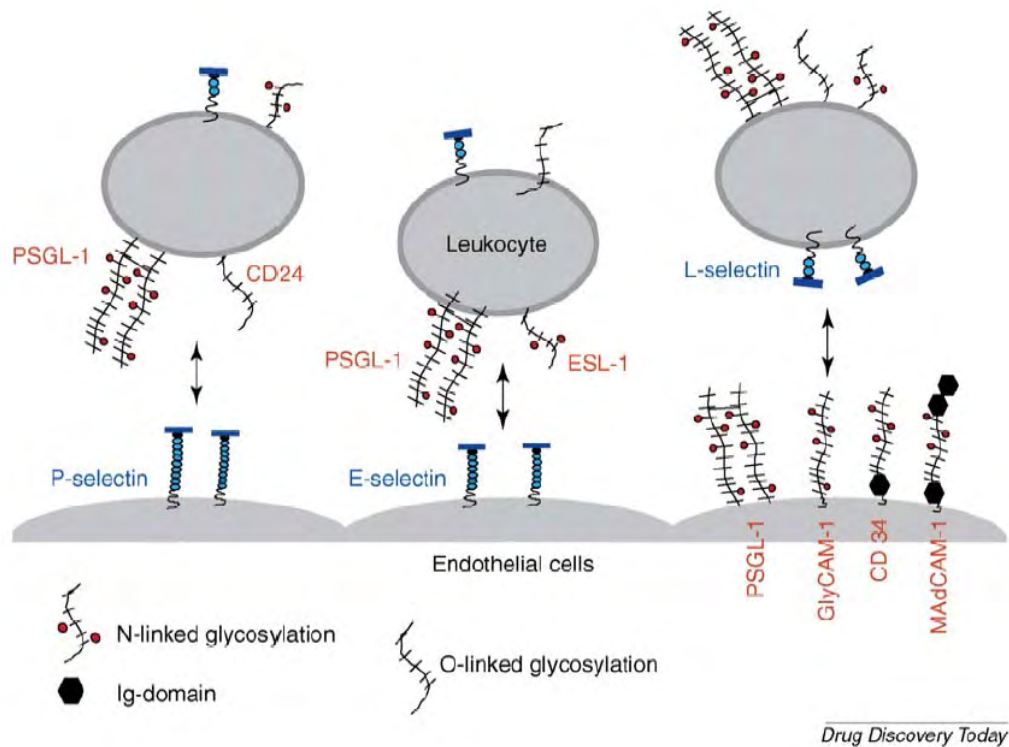


Figure 5. Interactions between selectins (blue) and their ligands (red) induce leukocyte tethering and subsequent rolling on activated endothelium. Activated leukocytes express N-glycosylated PSGL-1 and alternative O-glycosylated CD24 or N-glycosylated E-selectin ligand-1 (ESL-1) which interact, respectively, with P- and L-selectin on the endothelium. L-selectin expressed on leukocytes can also induce primary tethering, however at a lesser degree than the other family members. L-selectin elicits primary tethering through interactions with ligands PSGL-1, glycosylation dependent cell adhesion molecule 1 (GlyCAM-1), CD34 and mucosal vascular addressin cell adhesion molecule 1 (MadCAM-1) on endothelium. (Adapted from Kneuer, C, drug discovery today, 2006)

1.1.3. Integrins

The integrins are a diverse family of divalent cation-dependent membrane glycoproteins. They bind mainly to ECM molecules and cell surface proteins including IgSF members, although in some circumstances they also bind to soluble proteins (Takada et al., 2007). The composition and morphology of integrin mediated adhesion events, including focal complexes, focal adhesion, immunological synapses and fibrillary adhesion, vary greatly depending on the cell type, ECM and integrin. Integrins play an

important role in a variety of cellular functions, such as adhesion, survival or apoptosis, proliferation, motility and differentiation (Berrier and Yamada, 2007). These cellular events are important for vital processes such as development, vasculogenesis, immune responses and the integrity of the skin. They also have a critical role in immunological diseases and tumour progression.

Integrins are heterodimers composed of an α and β subunit, each of which contains a large extracellular domain, a single transmembrane domain and a short intracellular domain (Figure 6). The N-terminal regions of the α and β subunits interact non-covalently to form a globular ligand binding head piece attached to the membrane through leg-like α and β tailpiece regions (Shapiro et al., 2007). The α and β subunits show no structural homology.

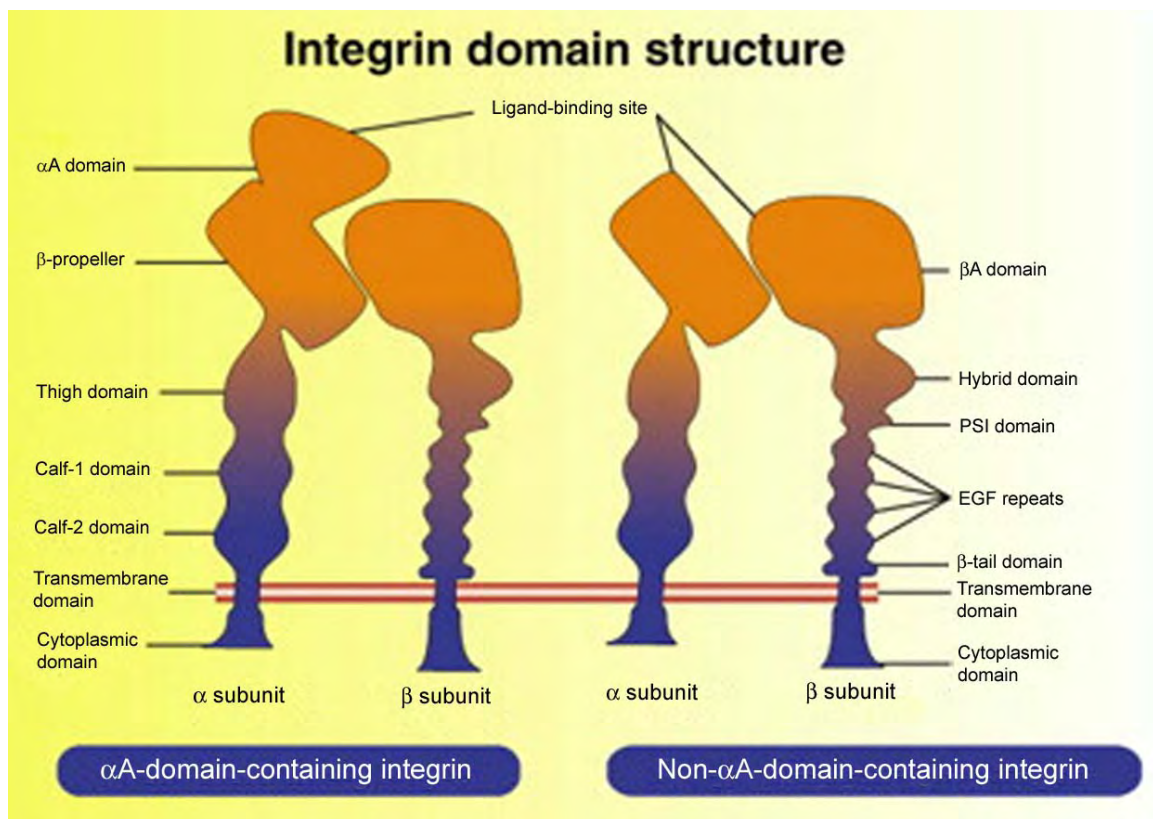


Figure 6. Schematic presentation of integrin structures. Integrin family members can be divided into two groups based on the structure of the α subunit, based on the presence or lack of a von Willebrand factor type A domain (vWFA), also commonly called the α A or α I (I for inserted) domain. (Adapted from Humphries JD et al., J Cell Sci, 2006)

Mammals express 18 different α subunits and 9 different β subunit and these can combine to form at least 24 different integrins, with distinct or overlapping ligand binding profiles (Figure 7). Cells commonly express several integrins that recognise the same ligand. Some integrins have a very limited expression pattern, such as $\alpha\text{IIb}\beta\text{3}$ in platelets, while others are widely distributed, such as $\alpha\text{V}\beta\text{3}$ in the endothelium. Integrin family members can be divided into two groups based on the structure of the α subunit, where it either contains or lacks an extra von Willebrand factor type A domain (vWFA), also commonly referred to as the αA or αI (I for inserted) domain (Smith, 2008). The αA domain is the major ligand binding site in this subfamily, while the integrins that lack the αA domain interact with their ligands primarily through an I-like domain in the β subunit. The αA and the β I-like domains contain metal ion-dependent adhesion sites (MIDAS) for which divalent cations are required for ligand binding. Integrin ligands compose a large and diverse group of molecules, however they can be divided into four main classes based on structural features of the integrin-ligand binding. We will not describe in detail the integrin-ligand binding sub-classes but these have been reviewed recently by Humphries, J., et al. (Humphries et al., 2006) and are illustrated in figure 7. ECM molecules including collagen, laminin and thrombospondin are dominant integrin ligands, however IgSF members such as ICAM and VCAM also interact with integrins. It is believed that ligand binding induces integrin clustering into large adhesive units.

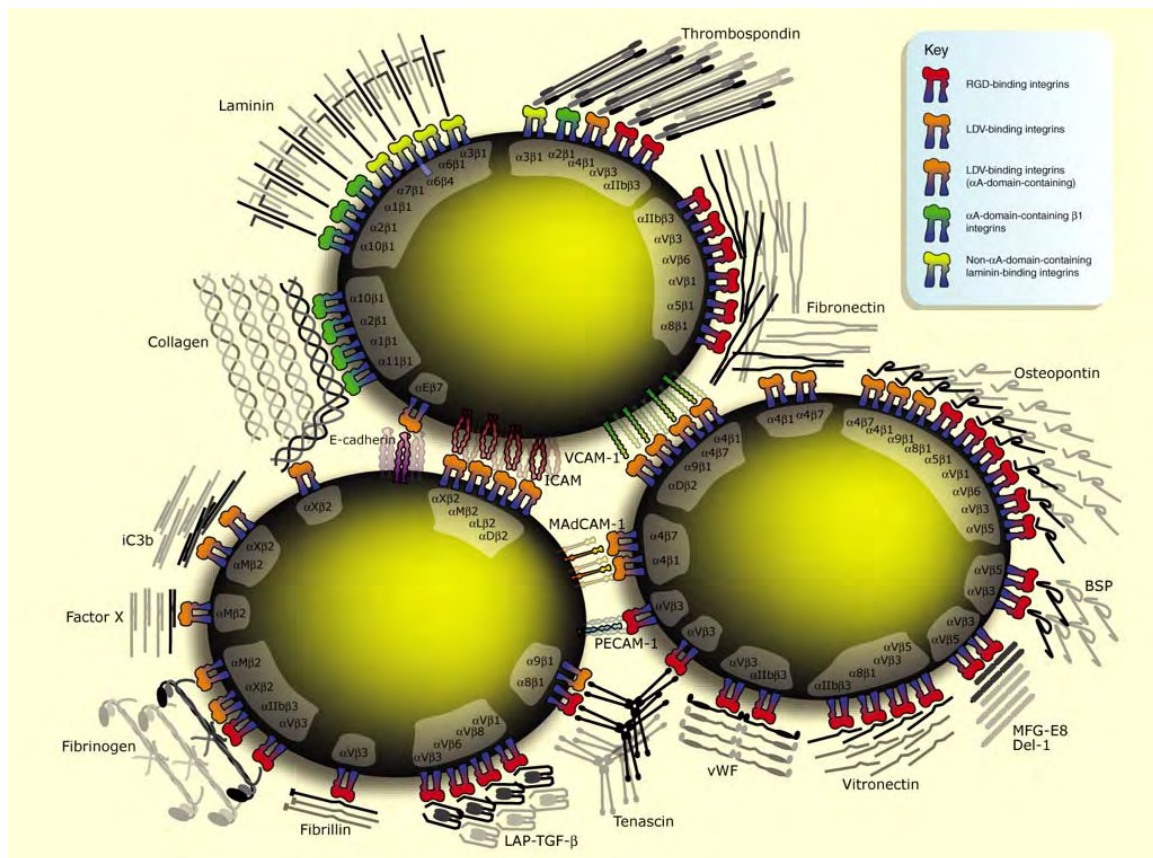


Figure 7. Presentation of the currently identified 24 mammalian integrins. The integrins have been presented on hypothetical surfaces and the best validated ligands are illustrated. The integrins have been divided into four major groups based on the structural features involved in ligand binding. These are presented with four different colour codes in the right upper corner. Please note that LDV-binding integrins have been further divided into integrins with or without the αA -domain, but they belong to the same group based on ligand binding and have the same colour code in the illustration. (From Humphries, JD, et al. J Cell Sci 2006)

Integrins have no intrinsic enzymatic activity, but they transmit signals from the surrounding into the cell by clustering kinases or adaptor proteins that form physical links with the cytoskeleton or signalling molecules (Avraamides et al., 2008). These clusters are intricate and involve many proteins, for instance focal adhesions where integrins are the central players can include up to a 100 different proteins (Lock et al., 2008). Integrins can mediate signals either from the outside into the cell, termed outside-in signalling or from the inside of the cell to the exterior, termed inside-out signalling. The cytoskeleton controls affinity and avidity of the integrin ectodomain and thus modulates the ECM or surrounding cells, and integrin binding to the ECM or surrounding cells changes the shape and composition of the cytoskeleton beneath, as shown in figure 8. In an inactive

state the integrin heterodimer is bent towards the membrane and the cytoplasmic domains are tightly together, while activation results in the elongation of the dimer and separation of the cytoplasmic domains. Upon activation through ligand binding the activated integrin binds enzymes including focal adhesion kinase/c-Src and the small GTPases Ras and Rho and adaptors including Cas/Crk and paxillin. Generally, the overall output is remodelling of the cytoskeleton, a process which is important in many cellular functions. A key player in integrin inside-out signalling is the actin binding protein talin (Arnaout et al., 2007). Stimuli from cell receptors, including, cytokines, chemokines and growth factors, activate the recruitment of talin to the plasma membrane where it binds to the cytoplasmic tail of the integrin β subunit and causes the dissociation of the cytoplasmic α and β subunits leading to an activated form of the integrin. In this activated form the integrin has a higher ligand affinity and talin recruits proteins such as paxillin, vinculin and α -actinin to strengthen the integrin-cytoskeleton interaction. Hence, integrins provide a bi-directional conduit for mechanochemical information across the cell membrane, providing a major mechanism for connecting the intracellular and extracellular compartments.

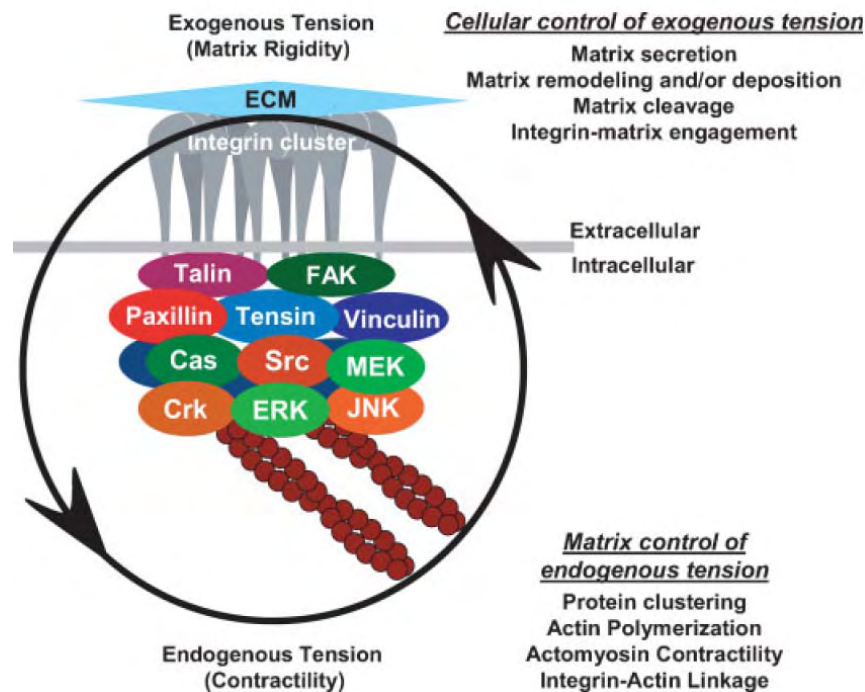


Figure 8. Integrins signal bi-directionally between the intra- and extracellular compartments, providing a link between extracellular and intracellular tension, a feature that is essential for cell migration and tissue

organisation. Integrin binding to the ECM triggers remodelling of the actin cytoskeleton and actomyosin contractility, while endogeneous tension can directly or indirectly control exogeneous tension (matrix rigidity). (From Berrier AL, J Cell Physiol., 2007)

1.1.4. Immunoglobulin superfamily

The immunoglobulin superfamily (IgSF) represents one of the largest protein superfamilies in the human genome. The first discoveries leading to the establishment of the IgSF were made in the late 1970's. Currently, 765 members have been identified in human, 140 members in fly and 64 in worm (Brummendorf and Lemmon, 2001). Interestingly when the number of members of a specific protein family is correlated with the biological complexity of the organism, IgSF ranks at the very top (Aricescu and Jones, 2007). This is partly due to the expansion of the immune system, but it could also be a consequence of increased complexity of developmental processes. IgSF members have diverse biological functions involving cell-cell recognition, migration, tissue organisation and cell differentiation. The IgSF superfamily includes a vast amount of proteins with different functions such as cell surface receptors, antigen presenting molecules, cell adhesion molecules and certain cytokine receptors. They are most commonly associated with the immune system.

IgSF members are mostly single-spanning type I membrane proteins, however approximately 10 % of the members are anchored to the membrane through a glycosylphosphatidylinositol moiety. The extracellular region contains one or more Ig-like domains. Ig-like domains have been divided into four subsets, namely the V-set, C1-set, C2-set and I-set (intermediate) according to their sequence and structural homology to the Ig V (variable) and Ig C (constant) domains (Barclay, 2003). The V-set domain resembles the Ig V domain. The C1-set domain is similar to Ig C domain, while the C2-set domain is similar to the Ig V domain in structure and the Ig C domain in length. The I-set domain has sequence features of the C2-set and the V-set. However, sequence and structural variations exist between the Ig-like domains of different family members and their correct categorization is not always obvious (Smith and Xue, 1997). The Ig domains are composed of an antiparallel, Greek-key, β sandwich structure shown in figure 9. The V-domain has two additional β strands (C' and C'') compared with the seven stranded

topology of the C-domain. The domain often contains a conserved disulphide bond that links the two β sheet strands B and F, however this is not essential for the structure and many IgSF members lack this bridge. The primitive C2- and I-set domains are found throughout the animal kingdom and are widely expressed in cell adhesion molecules, whereas the C1-set domain is present only in species that somatically rearrange their antigen-receptor genes and is almost exclusively found in Ig, MHC antigens and beta-2-microglobulin and the V-set domain is either directly inherited or obtained through somatic rearrangement (Chretien et al., 1998). IgSF proteins may contain all of the types of Ig-like domains. Generally the IgSF proteins that contain multiple Ig-like domains have an N-terminal V-set domain, which is often directly involved in the adhesive interaction, followed by one or more C-set domains. Furthermore, diverse domains, most commonly the fibronectin type III (FNIII) domain, form linkers between the Ig-like domains and the plasma membrane, thereby influencing the length of the extracellular domain (Shapiro et al., 2007). The IgSF members are often glycosylated to a varying extent depending on the cell type and tissue. Knowing that the N-linked carbohydrate chains can be almost as large as the Ig domain it seems likely that they play an important role in ligand binding and protein stability. Some IgSF members, such as the Down syndrome cell adhesion molecule (DSCAM) and neurofascin, show extensive alternative splicing. DSCAM has over 38 000 isoforms produced by four splicing hot spots acting on the Ig-like domains. This feature is crucial for dendrite patterning in the peripheral nervous system (PNS). Furthermore, soluble forms of IgSF proteins are often generated through alternative splicing.

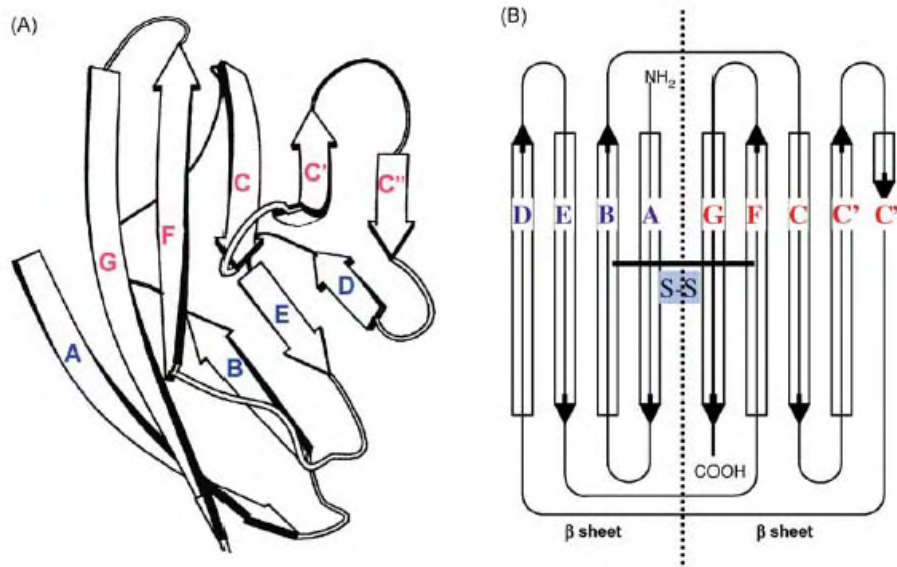


Figure 9. A schematic presentation of the Ig fold. a) The 3D model shows β strands as broad bands and loops joining them as thin tubes. b) The 2D image shows a V-domain composition with 9 β strands, while the C-domain lacks C' and C'' β strands. There can be some variations to the structure, e.g. the A strand can be split across both sheets. The conserved disulphide bond is indicated as SS between B and F strands. (From Barclay, AN., *Semin Immunol*, 2003)

The IgSF members have homophilic or heterophilic binding specificities, where heterophilic interactions involve other members of the IgSF, other cell adhesion molecules or cell membrane proteins such as growth factor receptors (Aricescu and Jones, 2007). Adhesive interactions can occur laterally on the membrane of the same cell (cis interactions) or between two opposite cells (trans interactions), for instance ICAM, VCAM and MadCAM can form hetero-trans adhesive interactions with Integrins, NCAM and L1 can form hetero-cis adhesive interactions with FGF (fibroblast growth factor) receptors and Nectins can form homo-cis and –trans adhesive interactions. However, even though a lot of focus has been placed on studying isolated extracellular cis and trans interactions, data now suggests that IgSF adhesive interactions are far more complex and involve assemblies of membrane and intracellular protein complexes. Frequently the cis and trans interactions form zippers or arrays making platforms at the cell-cell contact site (figure 10). Many of the cytoplasmic proteins that interact with the IgSF members link them to the cytoskeleton or promote cis interactions with other cell membrane proteins (Brummendorf and Lemmon, 2001).

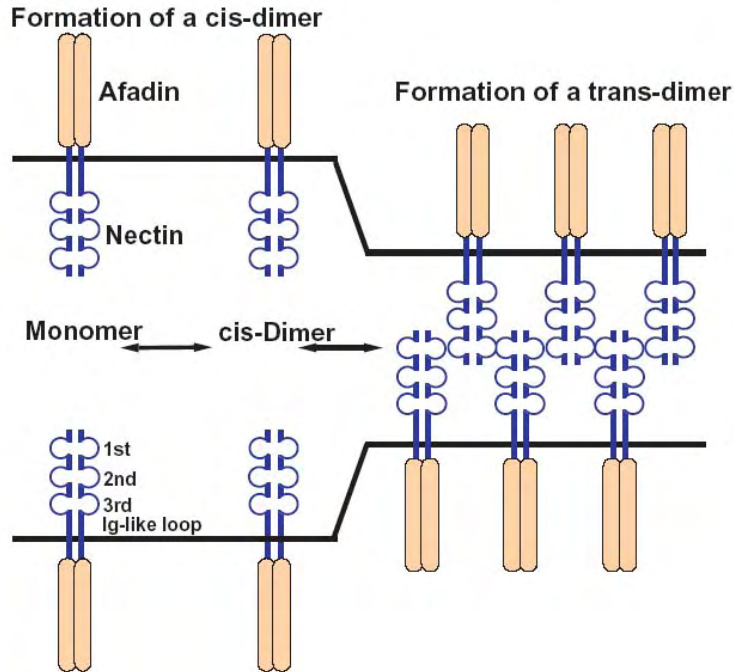


Figure 10. Example of Nectins forming homophilic cis and trans interactions. a) Schematic presentation of the adhesive properties of Nectin ectodomains. The N-terminal Ig-like domain is involved in trans adhesive interactions. The second Ig-like from the plasma membrane forms cis adhesive interactions in Nectins. Cis homo dimerisation is required for consequent trans homo interactions. Nectins bind to the cytoplasmic protein Afadin that links it to the actin cytoskeleton, a mechanism that is important for the adhesive properties. (From Takai, Y. and Nakanishi, H., J Cell Sci, 2003)

An IgSF sub-family named the CTX family was described by Chrétien I. *et al* following the discovery of novel proteins with high sequence and structural similarity to CTX, a cortical thymocyte marker in *Xenopus* (Chretien et al., 1998). They identified CTX homologous proteins in human and mouse that were termed CTH and CTM, respectively, in analogy to CTX. CTH is identical to human VSIG2 (V-set and immunoglobulin domains-containing protein 2). It is expressed in human stomach, colon, prostate, trachea, thyroid glands and at a lower level in bladder and lungs, while CTM is expressed in mouse stomach, prostate and at a lower level in colon. CTH and CTM have been suggested to play a role in tissue integrity. Other proteins that have been included in the CTX family are A33 antigen, CAR (Coxsackie and adenovirus 5 receptor), JAM (junctional adhesion molecules) proteins, ESAM (endothelial cell-selective adhesion molecule), BT-IgSF (brain- and testis-specific immunoglobulin superfamily protein) and CLMP (Car-like membrane protein, also called ASAM and ACAM) (Fok et al., 2007).

CTX family members are structurally composed of two extracellular Ig domains, including an N-terminal V-set domain and a cell membrane proximal C2-set domain, a single transmembrane domain and a cytoplasmic domain. They are generally differentially glycosylated on the extracellular domain depending on the cell type and activation state and they have several serine/threonine and tyrosine phosphorylation sites on the intracellular domains. The intracellular domain is the most divergent region and was reported to vary in length between 36 and 145 amino acids among family members.

A33 antigen, also called glycoprotein A33 (gpA33), is a single membrane spanning protein with an N-terminal V-set domain, followed by a C2-set domain and a highly polar intracellular domain (Heath et al., 1997). A33 is N-glycosylated at one or several of three potential sites and it is S-palmitoylated probably at four consecutive cysteine residues close to the transmembrane region in the intracellular domain (Ritter et al., 1997). The latter could be important in membrane tethering and consequently protein regulation through trafficking into endocytic vesicles. The theoretical molecular weight of A33 is approximately 33 kDa while that observed is 40 to 45 kDa, probably due to N-linked glycosylation. In humans, A33 expression is highly restricted to the epithelial cells of the small and large intestine, where it is specifically localized at the sublateral cell surface. A33 differs from most other intestine-specific proteins in that it is expressed at equivalent levels within the small and large intestines, probably regulated by the intestine-specific homeobox transcription factor, CDX1 (Johnstone et al., 2002). A33 is considered a cell adhesion molecule due to its predicted structure as well as its basolateral expression, however its functions remain unknown.

The JAM family proteins are expressed at the cell junctions of epithelial and endothelial cells as well as on the cell surface of leukocytes, platelets and erythrocytes (Mandell and Parkos, 2005). They are involved in functions including tight junction assembly, leukocyte adhesion and transmigration, platelet activation and angiogenesis. The JAM family includes JAM-A (also called JAM, JAM-1, 106 antigen, F11R), JAM-B (also called JAM-2, VE-JAM, hJAM2, mJAM-3), JAM-C (also called JAM3, hJAM3, mJAM-2) and the more recent members JAM4 and JAML (JAM-like, also called AMICA1). JAM-A, -B and -C share roughly 35 % sequence homology and are more closely related to each other than to any other IgSF, while JAM4 and JAML are less closely related to JAM-A, -B and -C with roughly 15 % sequence homology. Based on

sequence homology JAML resembles CAR and ESAM as closely as JAM-A, -B and -C. JAMs are single membrane spanning proteins with two Ig-like domains and generally a short cytoplasmic domain which contains a PDZ (postsynaptic density 95/disc-large/zonula occludens-1) domain binding motif and phosphorylation sites. There is some controversy about the sub-classification of the two Ig-like domains in the different JAMs (Mandell and Parkos, 2005), but generally they are reported to have an N-terminal V-set domain followed by a C2-set domain (Weber et al., 2007). JAM proteins are N-glycosylated but the significance of the glycosylation has not been determined. The cytoplasmic domains of JAM-A, -B and -C are short and comprise approximately 40 amino acids, while those of JAM4 and JAML are significantly longer with 105 and 98 amino acids, respectively. The crystal structure of the JAM-A extracellular domain has been resolved and it demonstrates that the protein has a bent structure, as shown in figure 11. It forms homo-cis complexes through a dimerisation motif on the V-set domain, which might be a prerequisite for the subsequent homo-trans interactions. Apart from homo interactions JAM family members also form hetero-cis and -trans interactions with other JAMs or CAMs, such as integrins.

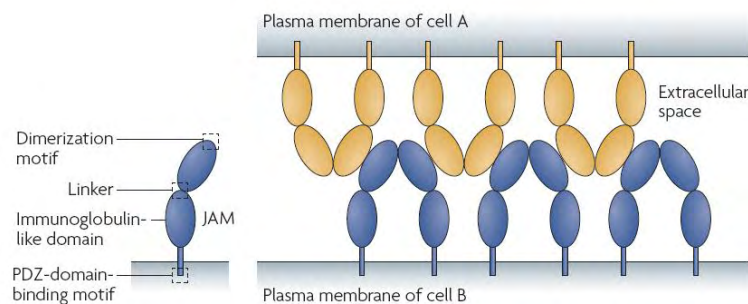


Figure 11. A schematic presentation of the structure of JAMs and a model for JAM homophilic adhesion. A) JAMs are composed of two Ig-like domains, a single transmembrane domain and an intracellular domain containing a PDZ-domain binding motif. A linker sequence between the two Ig-like domains confers a bent structure to the JAM extracellular domain. The N-terminal Ig-like domain contains a dimerization motif. B) In this proposed model for JAM homophilic interactions it forms a cis dimer forming an inverted "U" shape and consequently interacts to form a trans dimer. (From Weber, C. et al, Nat Rev Immunol, 2007)

JAM-A appears to be most widely expressed as it is found on endothelial and epithelial cells of liver, kidney, pancreas, heart, brain, lymph nodes, intestine, lungs, the placenta and vascular tissue as well as on circulating cells, such as platelet, monocytes,

lymphocytes and neutrophils. JAM-B is expressed on endothelial cells primarily in heart, lymph nodes, brain and kidney. JAM-C is expressed on leukocytes and endothelial cells, in intestine, lymph nodes, testis, liver, placenta and brain. JAM4 expression has been detected on mouse epithelial cells and JAML expression on human leukocytes, but their expression patterns have not been extensively studied. JAMs stabilize intracellular junctions and regulate the permeability of endothelial and epithelial cell layers. They are associated with the TJs and could play a role in recruiting specific proteins to this location through their PDZ domain binding motif. JAMs bind to various TJ associated PDZ domain containing proteins, including ZO-1 (zona occludens 1), AF-6 (afadin-6), CASK (calcium/calmodulin-dependent serine protein kinase), PAR-3 (partitioning defective 3) and MUPP-1 (multi-PDZ domain protein-1). These proteins are regulators of the actin cytoskeleton and hence can be important for the TJ assembly. There is evidence to suggest that the localisation of JAMs to the TJs is controlled by phosphorylation of their intracellular domains. The role of JAMs in the adhesion and paracellular permeability of the endothelium has been extensively studied in the context of inflammation. They play a crucial role in the recruitment of platelets and leukocytes to the endothelial membrane and the transmigration of the leukocytes through the membrane. JAMs on the surface of endothelial cells interact in a homo- or heterophilic manner with JAMs on leukocytes and platelets and with leukocyte integrins to induce adhesion and transmigration, as shown in figure 12. The importance of JAM controlled leukocyte transmigration through the endothelium is highlighted in the dysregulation of the blood-brain-barrier in multiple sclerosis patients (Padden et al., 2007). JAML was only recently discovered and has not yet been well characterised, but it could also play a role in leukocyte transmigration through the endothelium (Moog-Lutz et al., 2003).

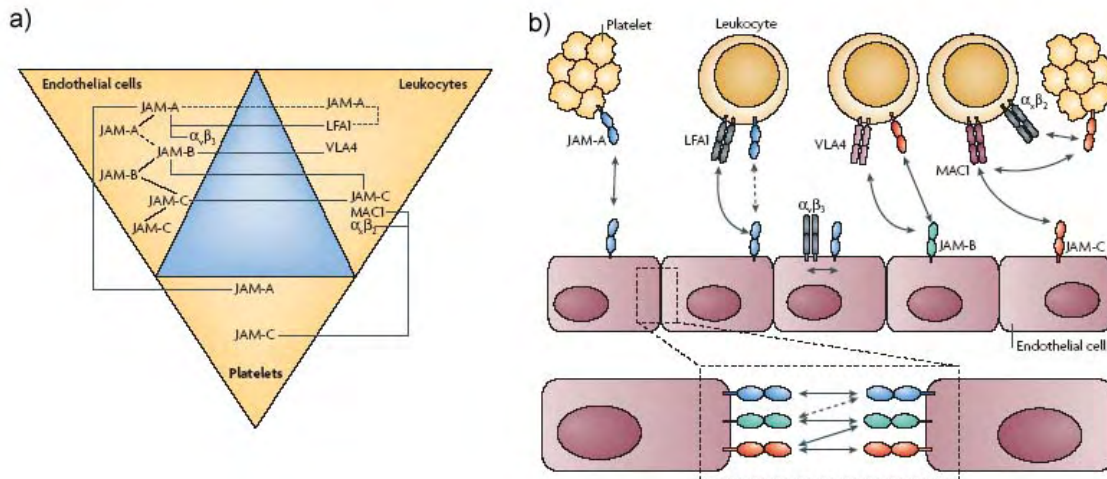


Figure 12. JAM cellular expression and extracellular ligands. A) At endothelial-cell junctions, JAMs undergo both homophilic and heterophilic interactions. JAM interactions that have been reported in the published literature are indicated with solid lines and predicted interactions are indicated with dashed lines. JAMs on endothelial cells also bind integrins expressed on leukocytes. The integrin lymphocyte function-associated antigen 1 (LFA1) interacts with JAM-A, very late antigen 4 (VLA4) interacts with JAM-B, and MAC1 interacts with JAM-C. b) Leukocyte binding to the endothelium is mediated by integrins and JAM interactions. JAM-A homophilic interactions mediate platelet adhesion to the endothelium and JAM-C heterophilic interactions with integrins mediate platelet adhesion to leukocytes. (From Weber, C. et al, Nat Rev Immunol, 2007)

JAM proteins also play an important role in angiogenesis (Naik et al., 2003a). The complex process of angiogenesis, i.e. the sprouting of new blood vessels, is important for normal growth and development but it occurs only under very specific conditions in an adult. This process includes the disruption of the endothelial cell contact with the ECM and the neighbouring cells, interplay between the endothelial cells, blood cells and smooth muscle cells, proteolytic release of matrix-bound angiogenic factors and consequently cell migration, proliferation and establishment of new blood vessels. Blood vessel repair is a similar process. JAM-A has been shown to associate with $\alpha_v\beta_3$ integrin and to be necessary for $\alpha_v\beta_3$ integrin mediated endothelial cell spreading (Naik and Naik, 2006). Furthermore, it is required for bFGF induced endothelial cell proliferation, angiogenesis, MAPK activation and endothelial cell tube formation (Naik et al., 2003b). Recent studies in HEK cells indicate that homo-cis dimerisation is necessary for JAM-A mediated cell adhesion and migration and that JAM-A PDZ domain binding motif is required for these processes (Severson et al., 2008). An alternative study involving JAM-C expression in a lung carcinoma cell line demonstrates, that the protein is important in

the regulation of the cell tight junction adhesions versus migration properties (Mandicourt et al., 2007). Furthermore, a role of JAM-C in angiogenesis was demonstrated using an anti-JAM-C monoclonal antibody (Lamagna et al., 2005).

JAM4 was discovered only recently, in a study to identify MAGI-1 (membrane associated guanylate kinase protein with inverted arrangement of protein-protein interaction domain 1) binding partners (Hirabayashi et al., 2003). MAGI-1 is a membrane associated protein localised at TJs. JAM4 has a similar structure to the other JAM family members, however it was reported to have two V-set Ig-like domains instead of one V-set and one C2-set domain. It also has a longer intracellular domain as mentioned above. Both Ig-like domains are important for JAM4 homo-trans interactions while only the N-terminal Ig-like domain is required for homo-cis interactions (Tajima et al., 2003). JAM4 induces homophilic cell clustering in a calcium-independent manner. The protein shows highest expression levels in the kidney, while expression is also detected in the stomach, small intestine, muscles, liver, bone marrow stem cells and testis (Nagamatsu et al., 2006). It is expressed both on the apical surface as well as at the TJs of kidney and small intestine epithelial cells and might be primarily targeted to the apical membrane and subsequently recruited to the TJs via currently unidentified interactions. JAM4 could be an important regulator of the integrity and permeability of the epithelium, since it reduces the paracellular permeability in a monolayer of COS cells. It could also be an important player in epithelial-to-mesenchymal transition (EMT) of cells, namely from a polarized non-migratory to a fibroblast-like migratory cell morphology. This mechanism is important in tissue morphogenesis and remodelling. Hepatocyte growth factor is an important regulator of cellular changes associated with EMT, such as proliferation, motility, remodelling of cell junction and formation of branching tubules. JAM4 enhances HGF-mediated branching (formation of tubular structures by cells in a 3D matrix) and cell motility, probably through a Rac dependent pathway (Mori et al., 2004). Likewise, TGF β (transforming growth factor β) induces EMT by enhancing cell migration, for example in fibrosis after tissue injury (Willis and Borok, 2007), and this process is associated with the redistribution of JAM4 in epithelial cells. The specific cell surface expression of JAM4 during EMT is suggested to be induced by intracellular binding of the LNX1 (Ligand-of-Numb protein X1) isoform, LNXp70, to the PDZ domain on the C-terminus of JAM4 (Kansaku et al., 2006). LNX1 forms a tripartite complex with JAM4 and Numb and thereby mediates JAM4 endocytosis, leading to cellular

redistribution or degradation. JAM4 is however not indispensable for normal tissue development, as JAM4 deficient mice show no evident defects (Nagamatsu et al., 2006).

ESAM could exert functions similar to JAM4 and JAM-A. ESAM is composed of a N-terminal V-set domain followed by a C2-set domain, a single transmembrane domain and a comparatively long intracellular domain (Hirata et al., 2001). It contains four potential glycosylation sites. The cytoplasmic domain consists of 120 amino acids long and contains proline rich sequences, a SH3 domain binding motif, a putative serine phosphorylation site and a type I PDZ domain binding motif. The C-terminal 20 amino acids show strong sequence homology with CAR. ESAM expression is strictly limited to endothelial cells, megakaryocytes and activated platelets (Nasdala et al., 2002). RNA level expression has been detected in the lungs, heart, kidney and skin, however a detailed analysis of ESAM tissue distribution still needs to be done. ESAM induces calcium independent homophilic cell clustering. Like JAM4, it binds to MAGI-1 in a PDZ-dependent manner, however ESAM does not bind the same PDZ domains on MAGI-1 (Wegmann et al., 2004). ESAM may be important for the recruitment of MAGI-1 to endothelial TJs. There is evidence to suggest that ESAM plays a role in vascular integrity as well as angiogenesis. ESAM is involved in the transendothelial migration of neutrophils, but not leukocytes (Wegmann et al., 2006). This process is not dependent on ESAM expression on activated platelets and ESAM does not play a role in neutrophil capture and firm adhesion. The protein seems to be involved in Rho GTPase activation, which affects the stability of endothelial contacts, leading to the hypothesis that ESAM could mediate neutrophil extravasation through this mechanism. It is strongly expressed in the developing vasculature of embryos, indicating that it could play a role in blood vessel formation. However, ESAM deficient mice do not show any defects in their vasculature and they develop and reproduce normally (Ishida et al., 2003). Nevertheless, ESAM deficient endothelial cells showed decreased migratory and angiogenic activity compared to wildtype cells. Furthermore, tumor growth, a mechanism that is dependent on vascularisation, was significantly retarded in ESAM deficient compared to wildtype mice. This suggests that ESAM mediated cell-cell contact contributes to the formation and function of blood vessels and ESAM could exert this role in a similar way to JAM-A described above.

1.2.Cell adhesion molecules in the CNS

1.2.1. The nervous system with a focus on the CNS – an overview

The human nervous system is an extremely complex structure with many still unanswered questions. Extensive studies are required to identify the functions and interconnections of the different cell types that constitute this system. This section will start with a general overview of the anatomy of the vertebrate nervous system and focus on a few structures that will become important later on in this work and will continue with a description of the main cell types within the nervous system with a focus of the cells of relevance to this study.

The vertebrate nervous system is divided into the central and peripheral nervous system. The central nervous system constitutes the brain and the spinal cord, while the peripheral nervous system comprises the sensory and motor neuron networks (figure 13a) (Book: Neuroscience. 2nd ed. Purves, Dale; Augustine, George.J.; Fitzpatrick, David; Katz, Lawrence.C.; LaMantia, Anthony-Samuel.; McNamara, James.O.; Williams, S. Mark, editors. Sunderland (MA): Sinauer Associates, Inc. c2001.). The peripheral motor neuron networks are subdivided into the voluntary somatic nervous system and the involuntary visceral (also called autonomous) nervous system. The visceral nervous system is further divided into the sympathetic nervous system that controls the “fight or flight” responses and the parasympathetic nervous system that controls the “rest and digest” responses. Morphologically the central nervous system is generally divided into seven basic parts: the spinal cord, the medulla, the pons, the cerebellum, the midbrain, the diencephalon and the cerebral hemispheres, as shown in figure 13b. The brain receives nerve impulses from the spinal cord and 12 cranial nerves (Guthrie, 2007).

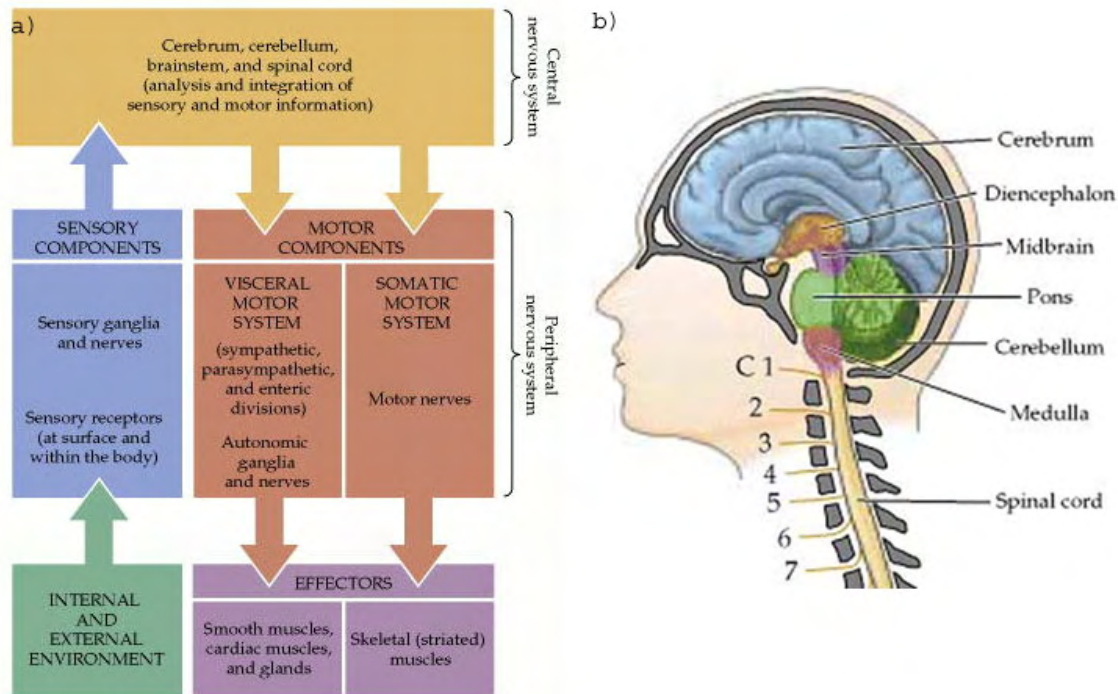


Figure 13. An overview of the nervous system and the morphology of the CNS. A) The nervous system is divided into the CNS and the PNS. Stimuli from the environment is transmitted through sensory neurons to processing circuits within the brain and spinal cord (the CNS), which in turn interpret their significance and send signals to peripheral effectors that move the body (somatic motor system) and adjust the workings of its internal organs (visceral motor system). (From Purves, D., et al. Neuroscience. 2nd ed, 2001.)

The spinal cord transmits signals between the brain and the body. It continues as an extension of the brain stem and traverses the spine all the way down to the coccyx, where it is attached through an extension of the pia mater. In a cross section of the spinal cord, the grey matter appears in the middle in the form of the letter H and is surrounded by white matter (see figure 14). The anterior “horns” of the grey matter H contain mostly somatic motor neuron cell bodies, the lateral “horns” (small projections on each side of the H) contain cell bodies of visceral motor neurons and the posterior “horns” contain mostly interneurons that synapse with sensory neurons. The cross-bar of the H is called the grey commissure, in the middle of which is a canal called the central canal. The central canal runs through the length of the spinal cord, contains cerebrospinal fluid (CSF) and connects with the fourth ventricle of the brain. The spinal nerves emerge along the length of the spinal cord in pairs, one from each side of the cord. On each side of the spinal cord the spinal nerves connect to the spinal cord

through so called roots, the ventral roots contain motor nerve axons that transmit information from the spinal cord to the skeletal muscles. The dorsal roots contain sensory nerve fibers transmitting information from the peripheral regions into the spinal cord.

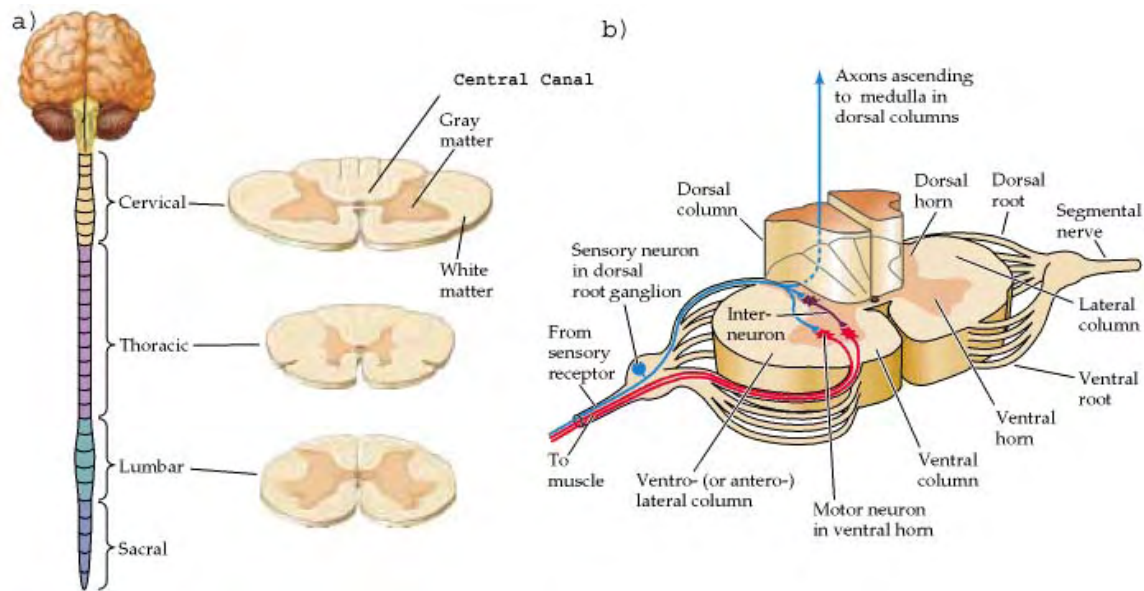


Figure 14. Morphology of the spinal cord. (A) Transverse sections of the cord at three different levels, showing the characteristic arrangement of gray and white matter in the cervical, thoracic, and lumbar cord. The central canal traverses the total length of the spinal cord in the middle of the grey commissure. (B) Diagram of the internal structure of the spinal cord. (From Purves, D., et al. Neuroscience. 2nd ed, 2001.)

The brainstem, consisting of the medulla, the pons and the midbrain, is a central player for brain functions as it is the target for sensory cranial nerves, the source of motor cranial nerves and a “thruway” for ascending sensory tracts from the spinal cord and the neck and for descending motor tracts from the forebrain. Therefore injury to the brainstem often leads to devastating combinations of functional deficits. The cerebellum in return is important for motor coordination, posture and balance and recent findings suggest that it might also be involved in certain cognitive processes (Apps and Garwicz, 2005). It is a highly convoluted hindbrain structure composed of a central white matter area surrounded by a three-layered cortex, containing five main cell types, including purkinje cells, granule cells, golgi cells, stellate cells and basket cells (Ramnani, 2006). The main information units of the cerebellum are the purkinje cells, which receive

information from the pontine nuclei and the inferior olive located in the brainstem through climbing fibers and parallel fibers. Interestingly, the human cerebellum contains ~50 billion neurons — roughly half of the total number of neurons in the brain (Zagon et al., 1977).

The midbrain occupies only a very small part of the human brain. It contains structures such as the reticular formation that passes on input from higher brain centers to the motor neurons, the substantia nigra that helps “smooth” out body movements and the ventral tegmental area that contains dopamine releasing neurons. The forebrain is divided into the diencephalon and the cerebral hemispheres. The two cerebral hemispheres are outlined by a layer of grey matter, the cerebral cortex. Functions such as speech, evaluation of stimuli, conscious thinking and control of muscle movement are processes in this layer. Within the cerebral grey matter lies the cerebral white matter. The white matter region is mostly composed of myelinated axons that connect the two hemispheres (associated fibers), connect gyri within hemisphere (commissural fibers) or connect the cerebrum to the spinal cord (projection fibers). The corpus callosum is the major assemblage of associated fibers allowing communication between the two cerebral hemispheres (Paul et al., 2007). The brain's complexity is largely determined by its connectivity, which is highlighted by the evolutionary increase in primate white matter volume (Schoenemann et al., 2005). In humans, the corpus callosum consists of over 190 million axons. There are several small structures of grey matter called basal ganglia (basal nuclei) that are located within the cerebral white matter. These regions are important for transmitting and modifying nerve impulses on their way from the cerebrum to the spinal cord. The diencephalon connects the cerebrum to the brainstem (midbrain and hindbrain). It contains structures such as the thalamus, which connects sensory nerves from the spinal cord to the cerebrum. The hypothalamus, which controls the visceral nervous system and regulates emotions, hunger, behaviour etc, is also found here.

The CNS tissue and fluid spaces are isolated from the blood by three main barriers, namely the blood-brain barrier (BBB) between blood and brain interstitial fluid (ISF), and the choroid plexus and the arachnoid epithelium between blood and the CSF (Figure 15). About 15 % of the brain volume consists of ISF that surrounds the neurons and glia (Segal, 2000). It has a similar composition to plasma, but with lower protein content, and lower K^+ and Ca^{2+} concentration and higher Mg^{2+} concentration. ISF homeostasis, which

is largely regulated by the BBB, is essential for proper nerve function. The BBB is composed of brain capillary endothelial cells surrounded by basal lamina, astrocytic perivascular endfeet, pericytes, microglia and neuronal processes (Abbott et al., 2006). The continuous endothelium of the brain capillaries has specific properties compared to non-BBB microvessels. It is less permeable due to complex tight junctions between the endothelial cells and hence forces any traffic through the endothelium to take a transcellular route. Small gaseous molecules such as O_2 and CO_2 and small lipophilic agents can diffuse freely through the lipid membranes, but the other molecules are transported through specific transport systems including enzymes, receptors and other carriers. Astrocytes at the BBB vicinity are important inducers of these functions. They can enhance the TJ assembly, the expression and localisation of transporters, such as PgP (P-glycoprotein) and GLUT1 (Glucose carrier), and enzymatic functions.

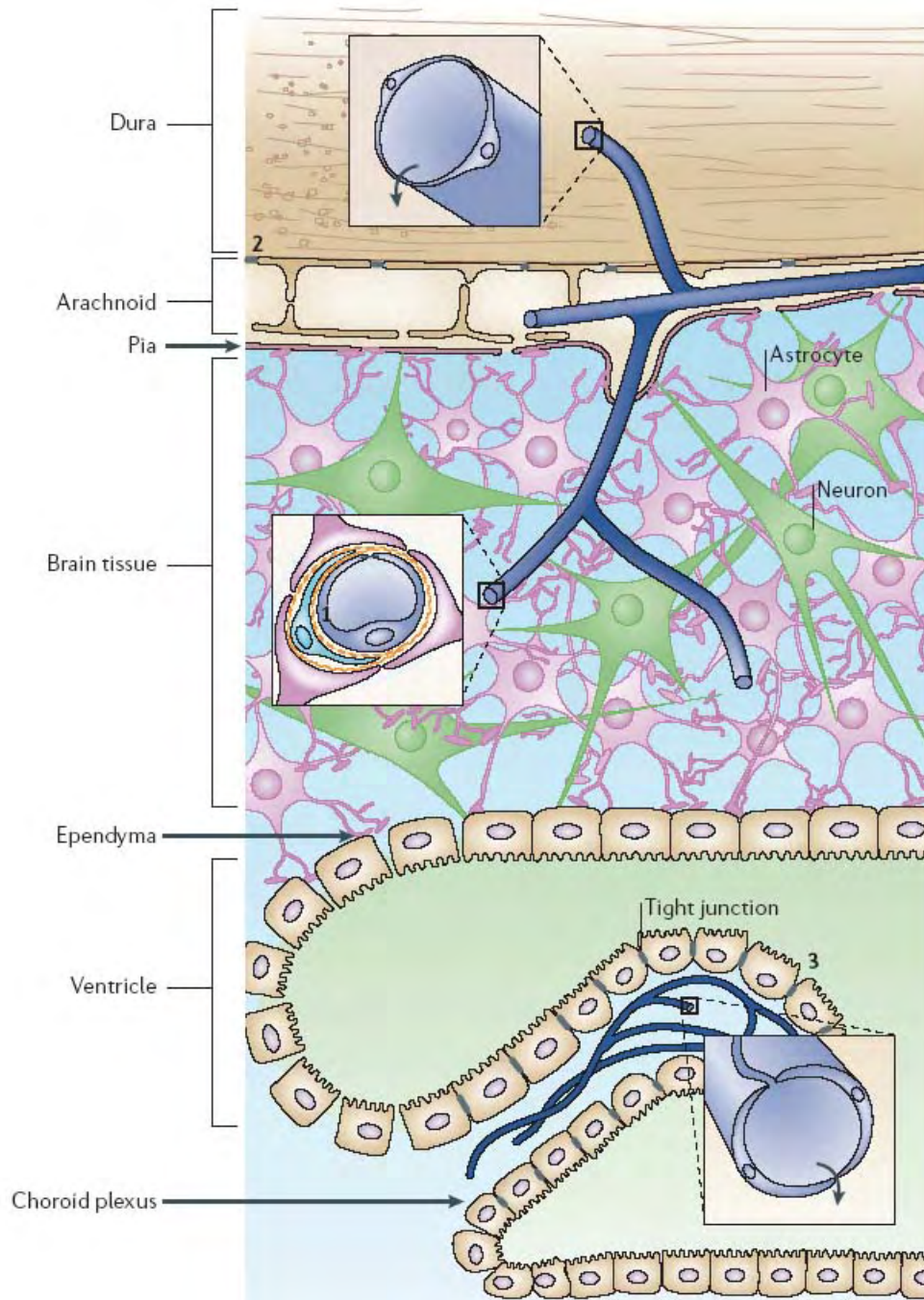


Figure 15. Barriers between blood and the interstitial fluid (ISF) or the cerebellar fluid (CSF). There are three main barriers, including (1) the blood-brain (or ISF) barrier, (2) the arachnoid epithelium which forms a CSF-blood barrier and is a major site for CSF drainage into venous blood and (3) the choroids plexuses which form the blood to CSF barrier and are a major site for CSF production. (From Abbott, NJ, Nat Rev Neurosci, 2006)

The choroid plexus in the ependymal layer and the arachnoid epithelium in the meninges constitute the barrier between the blood and the CSF. The main functions of the CSF, which takes up about 10% of the volume of the brain, are to cushion the brain, distribute nutrients and remove waste products and to provide a chemically stable environment (Praetorius, 2007). The CSF is found in the CNS compartments that are separated from the ISF compartments, which surround the neurons and neuroglia. The CSF runs through a system of connected cavities that include the ventricular system, the central canal of the spinal cord and the subarachnoid space (figure 16). The ventricles and the central canal are surrounded by ependymal cells, which are specialised glial cells with epithelial properties (Bruni et al., 1985). The choroidal ependyma constitutes the blood-CSF barrier and produces the majority of the CSF (Segal, 2000). These cells are amongst the most potent secretory cells in the organism. Components of the CSF return into the venous blood through arachnoid microvilli located in the Dural sinuses in the Dura mater. The Arachnoid epithelium is a part of the meninges, a continuous protective tissue layer that covers the brain and the spinal cord. The meninges is composed of three separate layers, namely from the outside inwards the dura mater, the arachnoid and the pia mater. The dura mater is thick and tough and presses against the inside of the vertebrae and the cranium. The arachnoid mater forms tubular structures (villi) that extend from it towards the inner layer, the pia mater, which closely follows the convolutions of the brain and the spinal cord.

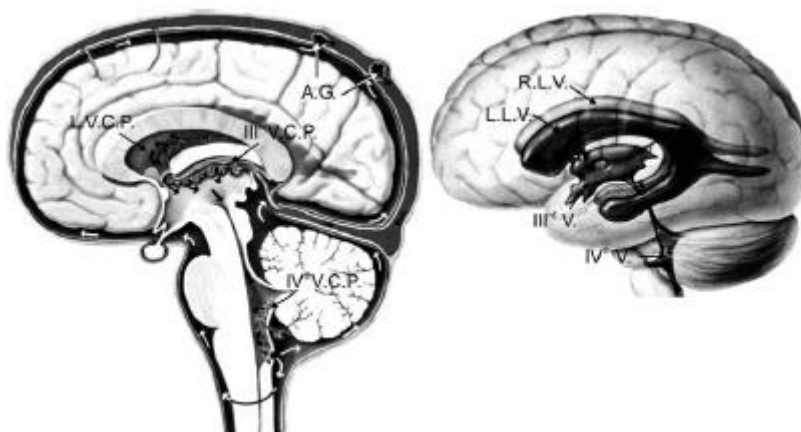


Figure 16. Cerebrospinal fluid containing compartments of the CNS. L.V.C.P. is the lateral ventricle choroid plexus; IIIrd V.C.P. is the third ventricle choroid plexus; IVth V.C.P.B. is the fourth ventricle choroids plexus; A.G. is arachnoid granulations. R.L.V. is the right lateral ventricle; L.L.V. is the left lateral ventricle; IIIrd V.

and IVth V. are the third and fourth ventricles, respectively. From Praetorius, J, Pfluger Arch-Eur J Physiol, 2007)

1.2.2. Cell types in the central nervous system – an overview

The nervous system is commonly divided into two main cell families, the neurons and the glia (figure 17). The human brain contains an estimated average of 100 billion neurons, each of which can potentially participate in thousands of connections (Purves, D., et al. Neuroscience. 2nd ed, 2001). Neurons form highly specialised circuits through which they transmit electrical signals. They can be of variable morphology, some with many intricately branched dendrites and others, even though a small minority, with no dendrites at all. The amount of input received by the neuron is in direct correlation with the complexity of its dendritic arbour. The dendrites and partly the cell body are the major sites for synapse formation with axon terminals of other neurons. Synapses are generated through intricate interactions of cell adhesion molecules and the electric pulses are transmitted by molecules secreted by the presynaptic terminal that bind to receptors on the postsynaptic receptors. The electrical impulse is then transmitted through the nerve axon to a synapse with another nerve cell. An axon can be from a few micrometers to about a meter (from the spinal cord to the foot).

It is estimated that glial cells occupy roughly half of the brain volume and that they outnumber the neurons by ten to one. At the time of their discovery it was believed that the main function of glial cells was to hold the neurons in place, hence the name glia from the latin word meaning glue. Since then, it has become evident that glial cells are crucial for proper nerve function. The glial cell family includes astrocytes, oligodendrocytes (OLs), ependymal cells and microglia (Figure 17) (Le et al., 2005).

Astrocytes are starshaped, multipolar cells. They form connections through microdomains such as lamellipodia and filopodia with adjacent neural synapses and surround the brain vascular epithelium with specialised end-feet (Volterra and Meldolesi, 2005). They are highly polyvalent cells involved in most functions in the CNS, including continuous monitoring and regulation of neural activity, regulation of cerebral blood flow,

neurogenesis, synaptogenesis, spatial positioning of OLs during development and attraction of microglia and lymphocytes to sites of injury. It is now clearly established that astrocytes also play important roles during brain injury or inflammation. The diverse astrocytic functions demonstrate the intricate relationship between the neurons and the glia in the CNS.

OLs are small with few narrow processes that change morphology during the course of their maturation. The main function of the OLs is to enwrap the axon in an isolating, lipid-rich membrane sheet, called the myelin sheet (Sherman and Brophy, 2005). The myelin sheets that enwrap the total length of the axon are periodically interrupted by the nodes of Ranvier. This axonal membrane structure allows membrane polarisation only at the nodes of Ranvier and hence results in a rapid, saltatory (from the Latin word *saltare*, to jump) nerve conduction. In the PNS, which lacks OLs, schwann cells have a similar function as OLs.

Microglia are smaller than the other glial cells and derived from hematopoietic stem cells. They are primarily scavenger cells with similar properties as macrophages and remove cell debris at sites of injury or during normal cell turnover. They also secrete inflammatory mediator substances such as cytokines to recruit other cells to the inflammatory site.

Ependymal cells, as previously mentioned, form the lining of the ventricles and the central canal, cavities containing CSF. Specialised ependymal cells at the choroids plexuses produce and secrete CSF.

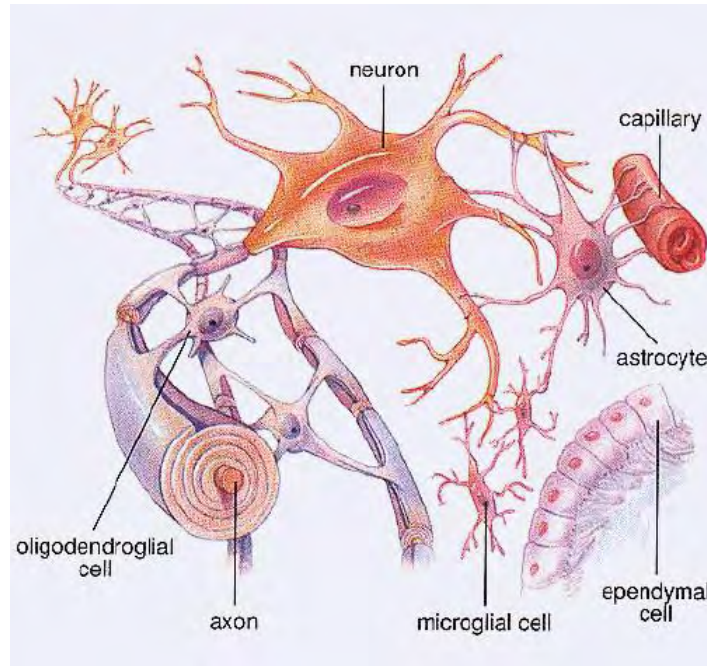


Figure 17. The main cellular components of the central nervous system. There are roughly ten times more glial cells in the human CNS compared to neurons. The glial cell family includes OLGs, astrocytes, microglia and ependymal cells.

1.2.3. Cell adhesion molecules in the CNS – general features

The complex structure and functions of the CNS depends on temporally and spatially regulated cellular interactions. CAMs in the CNS play a role in regulating such diverse events as growth cone guidance and motility, proliferation, axon guidance and fasciculation (when axons elongate alongside other axons, forming bundles or fascicles), contacts between neural cell bodies, axonal attachment to and autotypic junctions within OLGs, astrocytic connections adjacent to neural synapses and contacts to vascular epithelial cells (Maness and Schachner, 2007). Some of these interactions are illustrated in figure 18. In the mature brain, specific cellular adhesions are often correlated with histologically distinct features, such as cellular clustering into nuclear groups, cortical layers (eg, meninges), fiber tracts (eg, corpus callosum) and synaptic terminal fields (Shapiro et al., 2007).

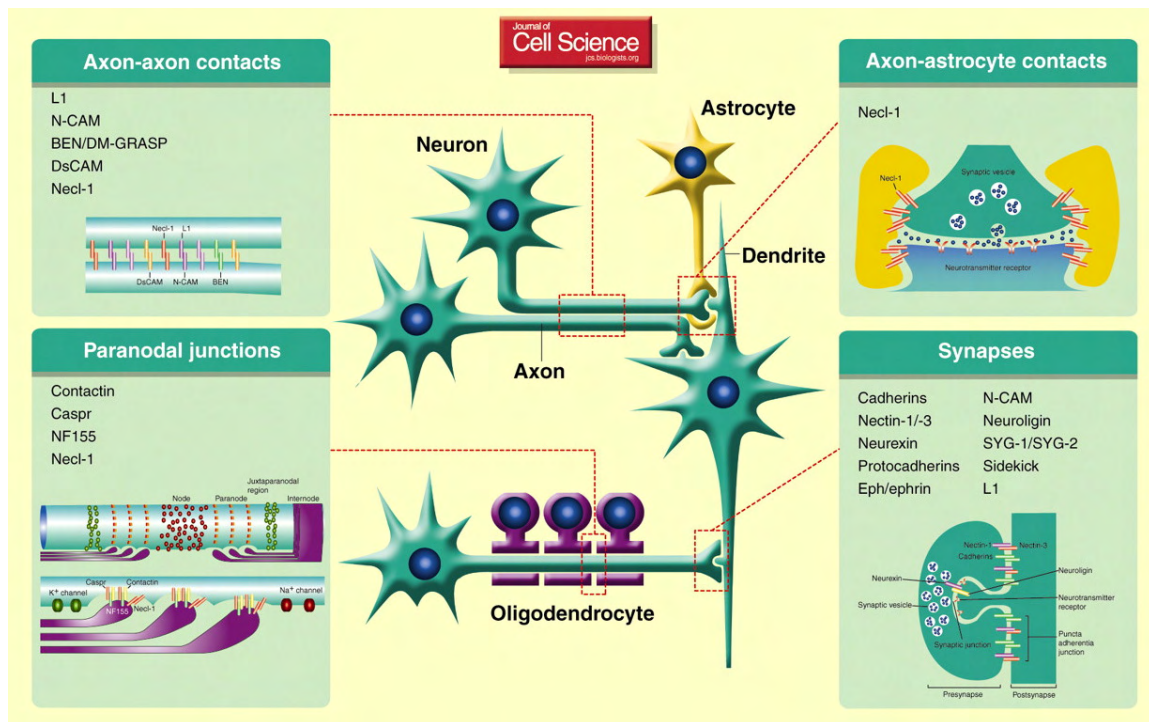


Figure 18. Schematic presentation of some of the major cell-cell interactions in the CNS. These include crosstalk between neural axons, neural synapses with surrounding astrocyte endfeet and OL-axolemma interactions leading to axon myelination. (From Sakisaka T, J Cell Sci, 2005)

Nerve synapses are specialised cell contact sites involving several CAMs. Synaptogenesis and maturation are mediated by cell membrane receptors, CAMs, signalling molecules and scaffolding proteins. Examples of the adhesive interactions at the neural synapse include contacts between neurexin and neuroligin, Eph and Ephrin and cadherin homo-interactions (Figure 18) (Dalva et al., 2007). Multiple cadherins are expressed in the brain and they could function as a key-lock mechanism, where they form adhesive interactions at the sides of the active synapse. EphB-Ephrin-B interactions have been extensively studied in synapses, they regulate adhesion versus repulsion in synaptogenesis. Neuroligin, an esterase-like-domain-containing single transmembrane protein on the presynaptic side, interacts with β -neurexin, a laminin-globular-domain containing single transmembrane protein located on the post-synaptic membrane. Neuroligins cluster through homo-cis interactions in the presynaptic membrane and then bind to and induces clustering of β -neurexin on the postsynaptic membrane. This leads to the recruitment of pre- and postsynaptic signalling and

scaffolding proteins and activates the synaptic machinery. Current data suggests that neuroligin- β -neurexin adhesion can lead both to excitatory and inhibitory synapse formation. Neurexins exist in over a thousand different isoforms generated by alternative splicing, and these proteins could play a role in specifying certain classes or types of synapses.

Adhesive and repulsive contacts between nerve axons play an important role in axon guidance and fasciculation. The best studied CAMs involved in these functions are L1 and NCAM (neural cell adhesion molecule) (Figure 18) (Sakisaka and Takai, 2005). L1-related molecules contain an extracellular region of six immunoglobulin-like domains and four to five fibronectin type III repeats, followed by a highly conserved cytoplasmic domain of ~110 residues. This CAM subfamily includes Nr-CAM, Ng-CAM, neurofascin, neuroglian, aBGP and CHL1. L1 interacts with integrins NCAM, TAG-1/axonin, contactin/F3/F11 and ECM molecules at its extracellular domain and connects to the actin cytoskeleton via ankyrin binding to its cytoplasmic tail. There are three NCAM isoforms, each containing five immunoglobulin-like domains and two fibronectin type III homologous repeats, followed in NCAM180 and NCAM140 by a transmembrane domain and in NCAM120 by a glycosylphosphatidylinositol (GPI) anchor that attaches it to the membrane. NCAM forms homophilic and heterophilic interactions with a variety of proteins, including FGFR, L1, TAG-1/axonin and heparin sulphate proteoglycans. Axon guidance and fasciculation mediated by NCAM seems to be primarily regulated by a polysialic acid (PSA) moiety attached to the protein. OL and astrocyte interactions with neurons are described below.

1.2.4. Oligodendrocyte maturation and cell adhesion molecules

OLs are generated from neuroepithelial stem cells at two main sites, the oligodendrocyte precursor cells (OPCs) nestled in the subventricular zone (SVZ) in the brain and a group of Sox10/Olig1-positive cells in the ventral spinal cord (Chen et al., 2007). From there they travel through the complex architecture of the brain to the site of axon myelination. Hence, in order to become myelinating OLs the cells undergo

extensive proliferation, differentiation and migration, mechanisms that involve growth factor mediated activation, remodelling of the cytoskeleton and, cell-cell and cell-ECM interactions. The main stages of OL differentiation are shown in figure 19 below.

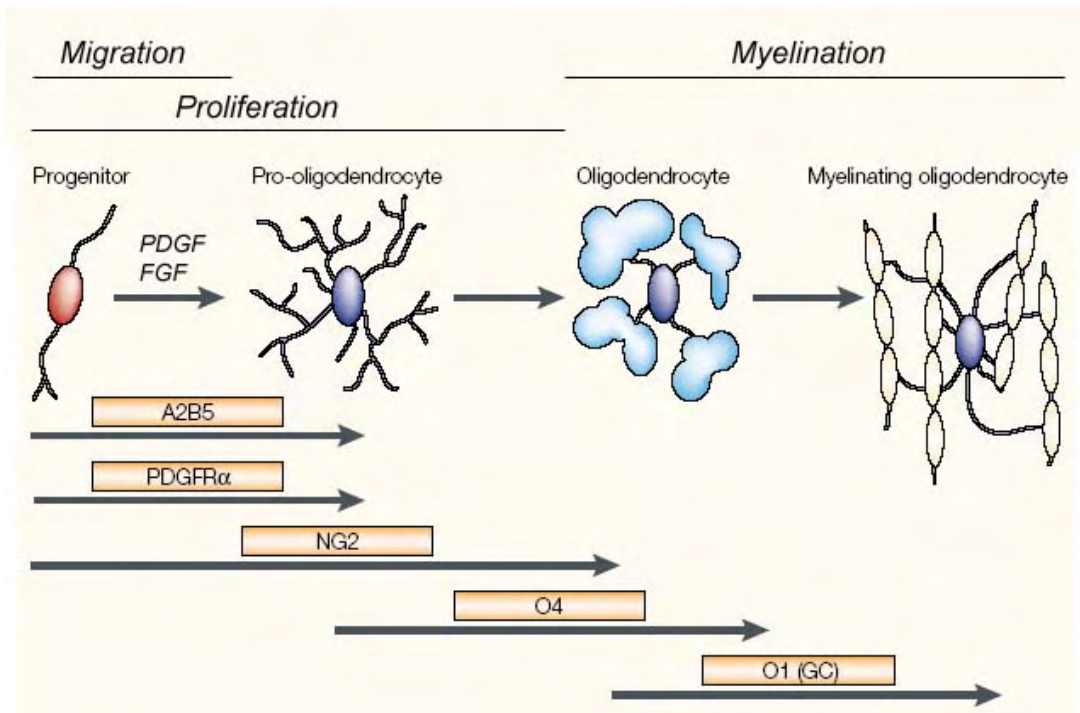


Figure 19. OLs undergo extensive morphological changes as they differentiate from bipolar OPCs to mature myelinating OLs. OLs at different stages of maturation can be identified through specific markers. The monoclonal antibodies A2B5 detects gangliosides, O4 detects sulfatides and glycolipids and O1 detects GalC (galactocerebroside). Other markers include PDGFR α (platelet derived growth factor receptor alpha) and the proteoglycan NG2. PDGF (platelet derived growth factor) and FGF (fibroblast growth factor) enhance OPC proliferation and migration. (Adapted from Zhang S, Nat Rev Neuro, 2001)

The OPCs generated at the SVZ migrate to populate different parts of the cortex (Richardson et al., 2006). The Sox10/Olig1-positive OPCs migrate radially to populate the spinal cord, a mechanism that is dependent on transcription factors olig1 and olig2 because olig1/olig2 double knockout mice lack spinal cord OLs (Ndubaku and de Bellard, 2008). OPC differentiation and the onset of myelination start at a late stage of embryogenesis and continue for several weeks in the rodent brain and for several years in the human brain. Furthermore, a continuous slow turnover of myelin persists throughout the lifetime of a mammalian brain, requiring constant regeneration of new

OLs (Ventura and Goldman, 2006). The ability of multipotent cells to differentiate into committed glioblasts and then further to mature glial cells requires a plethora of different trophic factors and/or morphogens, signalling receptors and transcription factors. Trophic factors and/or morphogens that have been shown to promote OPC differentiation include, PDGF (platelet derived growth factor), bFGF/FGF-2 (basic fibroblast growth factor), T3 (Triiodothyronine), Shh (Sonic hedgehog), cAMP (adenosine 3':5' cyclic monophosphate), retinoic acid and neuregulin. While PDGF and bFGF contribute to the proliferation and migration of OPCs, neuregulin is required for maintenance and survival, whereas cAMP and retinoic acid regulate the differentiation of OPC into more mature stages. The transcription factors involved in OPC differentiation form a very complex regulated interdependent chain (Nicolay et al., 2007). There has been a lot of ambiguity about which transcription factors act in this process and when and where they mediate their function. Now it is known that OPCs that are generated in different parts of the CNS are differentially regulated and that they probably have different properties before they all converge into the same function of myelinating axons (Kessaris et al., 2006). Neural basic helix–loop–helix (HLH; i.e., Olig1 and Olig2, Mash1), associated inhibitory HLH (i.e., Id2 and Id4), high-mobility group domain (i.e., Sox8, Sox9 and Sox10), and homeodomain (i.e., Nkx2.2) transcription factors have all been directly implicated in OL development. Out of numerous studies, the Olig and Sox groups are probably the best characterised and were shown to play a critical role in OPC differentiation.

Migratory OPCs are typically PSA-NCAM-positive (polysialic acid linked neural cell-adhesion molecule) cells that express a ganglioside recognized by the A2B5 antibody (Maness and Schachner, 2007). NCAM120 isoform is predominantly expressed by glial cells, while the other NCAM isoforms are expressed by neurons. The existence of α -2,8-linked polysialic acid on NCAM may decrease homo- and heterophilic NCAM interactions because of its highly negative charge and large hydration volume. It may also influence the ability of NCAM to interact with heparin and heparin sulfate, which present growth factors to their cognate receptors. These properties probably lead to the pro-migratory properties of PSA-NCAM. Several ECM components, such as laminin, fibronectin and vitronectin have been shown to promote OPC migration, while collagen IV and tenascin-C function as barriers for migration (Jarjour and Kennedy, 2004). The pro-migratory mediators do not show a concentration gradient dependent increase in migration and are therefore termed short-range cues. Both fibronectin and vitronectin are expressed along white matter tracts during development, while laminin-1 is restricted to

basement membranes and capillary walls in both the developing and mature CNS. Long-range attractive cues for OL migration include PDGF (platelet derived growth factor) and bFGF (basic fibroblast growth factor). Interestingly the effect of these two growth factors on OL migration is not cumulative, suggesting that they might target similar pathways. OPCs express high levels of PDGFRA through which they respond to PDGF secreted by surrounding neurons and astrocytes. PDGF-A knockout mice show a severe reduction in the numbers of OPCs and dysmyelination, which could be due to a defect in both proliferation and migration of these OPCs (Fruttiger et al., 1999). OPCs in the embryonic spinal cord and optic nerve express the netrin receptors DCC and Unc5H1 and Netrin-1 is suggested to be a long-range repellent cue for OL migration since a netrin-1 gradient *in vitro* repels OPC migration and in mice lacking either netrin-1 or DCC, OPC migration from ventral to dorsal spinal cord is impaired. OPCs also express a broad range of semaphorins, including semaphorin classes 3-7 and two neuropilins, which generally bind to semaphoring class 3 (Cohen, 2005). Different studies show either an attractive or repulsive effect of semaphorins on OPCs, depending on the tissue origin of the OPCs and the methods used, but clearly these proteins are involved in the regulation of OPC migration. Since OPCs can both respond and express semaphorins, their guidance is possibly regulated in an autologous and/or heterologous manner.

Factor regulating OPC migration presumably act either directly or indirectly on integrins, which have a key role in cell migration (Hood and Cheresch, 2002). Several integrins are expressed in OLs at different stages of maturation, these include $\alpha 6\beta 1$, $\alpha \nu\beta 1$, $\alpha \nu\beta 3$, $\alpha \nu\beta 5$, and $\alpha \nu\beta 8$. Accordingly, $\alpha \nu\beta 1$ integrin binds fibronectin and vitronectin and enhanced $\alpha \nu\beta 1$ integrin levels are correlated with OPC migration. Furthermore, PDGFRA and the $\alpha \nu\beta 3$ integrin interact in OPCs and proliferation at physiological PDGF-A levels (0.1 ± 1 ng/ml) requires $\alpha \nu\beta 3$ ligand binding (Baron et al., 2002). It has also been demonstrated that OSP/Claudin-11, a cadherin-type protein found in myelin tight junctions, forms a complex with OAP-1 (OSP/Claudin-11-associated protein) and $\beta 1$ integrin, and that this plays a role in OPC migration (Tiwari-Woodruff et al., 2001). At their leading edge, OLs form growth cone-like structures similar to axonal growth cones (Fox et al., 2006). These structures contain F-actin and at the leading edge microtubules project from the cell body along the process. They are highly dynamic structures that undergo outgrowth, retraction and branching, and accordingly contain GAP43 (growth-associated protein) and cofilin, which are regulators of the actin cytoskeleton and present in neuronal growth cones. These structures could be important to regulate the

direction of OPC migration as well as for making a primary contact with the axolemma. OPCs in cell culture proliferate in response to a number of different growth factors, including PDGF, FGFs, insulin-like growth factors (IGFs) and neuregulin (Baron et al., 2002). Proliferating OPCs do not differentiate and still have the potential to revert to a stem cell phenotype (neural precursor cell), however when they cease to divide they constitutively differentiate into myelin-forming OLs unless inhibitory cues such as the Notch signalling pathway is activated. Eventually, in the animal, the OPCs reach their target regions in the CNS, stop migrating and mature into myelinating cells and start expressing myelin proteins, proteolipid protein (PLP), MBP and MAG.

OL mediated axon myelination is a remarkable example of cell-cell adhesion and coordination. Each OL makes multiple processes, each of which myelinates distinct internodes, often on different axons. The amount of myelin sheets produced by one OL seems to be dependent on the diameter of the surrounding axons, e.g. OLs have fewer processes in tracts containing large myelinated fibers (Arroyo and Scherer, 2000). Furthermore, the thickness of the myelin sheet is also in direct relation with the axon diameter. The diameter of the axon divided by the diameter of the axon and the myelin sheet gives a value between 0.6 and 0.7 that is called the g-ratio. This value is generally constant with the exception of remyelinated axons where the myelin sheet is thinner than expected. The thickness of the myelin sheet seems to be largely regulated by the Neuregulin-Erb receptor system (French-Constant et al., 2004). There is still some ambiguity on how myelination is initiated, however possible factors include neuron produced effectors such as neuregulins, jagged and ATP. Recent evidence suggests that some of these effectors might act directly while others are transduced via astrocytes (Spiegel and Peles, 2006). Indeed ATP is shown to induce astrocytic secretion of LIF (Leukemia inhibitory factor) that in return stimulates OL myelination. These effectors probably act on the axon themselves to induce downregulation of inhibitory CAMs, such as NCAM and L1, and upregulation of CAMs, such as Caspr (Contactin-associated protein) and contactin, that interact with OL CAMs (Sherman and Brophy, 2005). In addition they promote OL myelin sheet formation and upregulation of CAMs in the OL that interact with the axon, such as the glial IgCAM, Nfasc155 (Neurofascin-155), shown in figure 20. More than 50 types of neurofascins have been identified to date. They are members of the L1 subgroup of neural IgCAMs and are implicated in neuron–neuron and neuron–glia interactions during nervous system histogenesis (Brummendorf and Lemmon, 2001). It is suggested that primary axolemma-OL interactions could involve

axonal Caspr and contactin binding to Nfasc155 on the OL process. This is followed by ankyrin-G mediated linkage of Nfasc186, Nr-CAM (Neural-glial related cell adhesion molecule) and voltage gated sodium channels to β IV-spectrin to form the nodes between the myelin segments. Hence, the contact between the OL membrane and the axolemma is probably necessary for the formation of the distinct protein clusters along these membranes. However, it has been demonstrated that myelination is not required for the clustering of the sodium channels in the CNS, however in the absence of myelination the nerve conduction rate is reduced. The membrane segment involved in the interaction between the myelin sheet and the axolemma has been divided in specific regions depending on the composition. These divisions include the paranodes adjacent to the nodes, followed by the juxtaparanodes on both sides of the internal segment, the internodes. The paranodes contain Caspr and contactin interactions with Nfasc155, the juxtaparanodes include Caspr-2 that binds to potassium channels Kv1.1 and Kv1.2 and their associated β 2-subunit, which are important for the dampening of the excitability of the myelinated fibers. The OL membrane at the juxtaparanodes also contains potassium channels, possibly to remove excess K⁺ that accumulates in the periaxonal space. The internodal regions do not seem to have distinct clustering of membrane proteins, but the paranodal proteins can also be detected as dispersed units on this membrane portion.

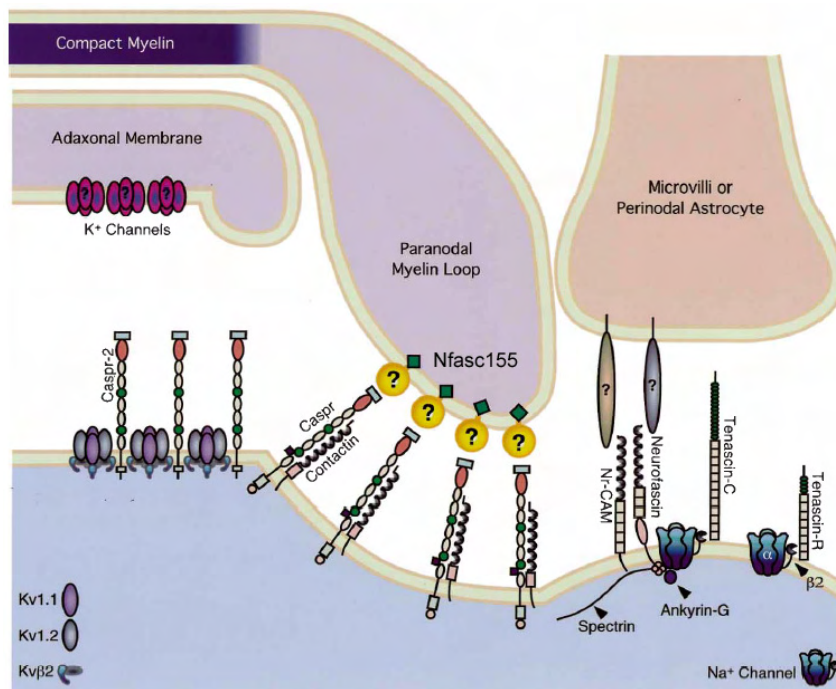


Figure 20. A schematic presentation of the cis and trans interactions at the nodes, paranodes and juxtaparanodes. Note that since the realisation of this picture Nfasc155 (Neurofascin-155) has been identified as the glial counter-receptor of Caspr (Contactin-associated protein) and Contactin at the paranodes. (Adapted from Arroyo E., Histochem Cell Biol, 2000)

The myelin sheet contains many different microdomains with distinct compositions and functions, these include lipid rafts, caveolae, tetraspanin-enriched domains and cell junctions, including tight, adherens and gap junctions (figure 21). The myelin sheet is a shovel-shaped myelin layer surrounded by a thicker, continuous cytoplasmic rim that is wrapped several times around the axon. Electron micrographs of vertical sections of the axon and overlying myelin sheet show major dense lines, which are the intracellular portions of the sheet, and intraperiod lines which are the intra-membraneous extracellular portion. The extracellular domains of cell adhesion molecules form regions where the intraperiod line is more or less thin, referred to as compact or non-compact myelin. Proteolipid protein (PLP) is the main protein of CNS compact myelin. It forms adherens junctions, has cadherin-like properties and can function as a Na^+ and Cl^- ion pump (Dyer, 2002). PLP interacts with MBP (Myelin basic protein) that possibly regulates its ion pump functions. MBP is a cytoplasmic protein and it is the second most abundant protein in the myelin sheet, hence it occupies the bulk of the intracellular space within the sheet. It exists in several isoforms with diverse post-translational modifications, which can have unique functional roles in specific microdomains and possibly a role in organising the microdomains. Interestingly, MBP mRNA has been detected at distal sites of OL processes indicating that it is produced locally. Non-compact myelin is seen at the paranodes as well as incisures, including the radial components and Schmidt-Lanterman incisures, which are radially arranged intralamellar non-compact strands that span the myelin sheet from the core to the outer surface. The radial components are largely composed of OSP (OL-specific protein)/Claudin-11 mediated tight junctions. OSP/Claudin-11 is a tetraspan membrane protein of the Claudin family and it is the third most abundant protein in the CNS myelin sheet. Additionally, radial components are enriched in cytoplasmic proteins CNPase (2',3'-cyclic nucleotide 3'-phosphohydrolase) which binds to actin, tubulin and 17 and 21.5 kDa isoforms of MBP, and the membraneous components cerebroside and cholesterol. The functions of the radial components remain to be elucidated, but it is suggested that they

play a role in the perfusion of the periaxonal space, especially removing potassium ions (Dyer, 2002).

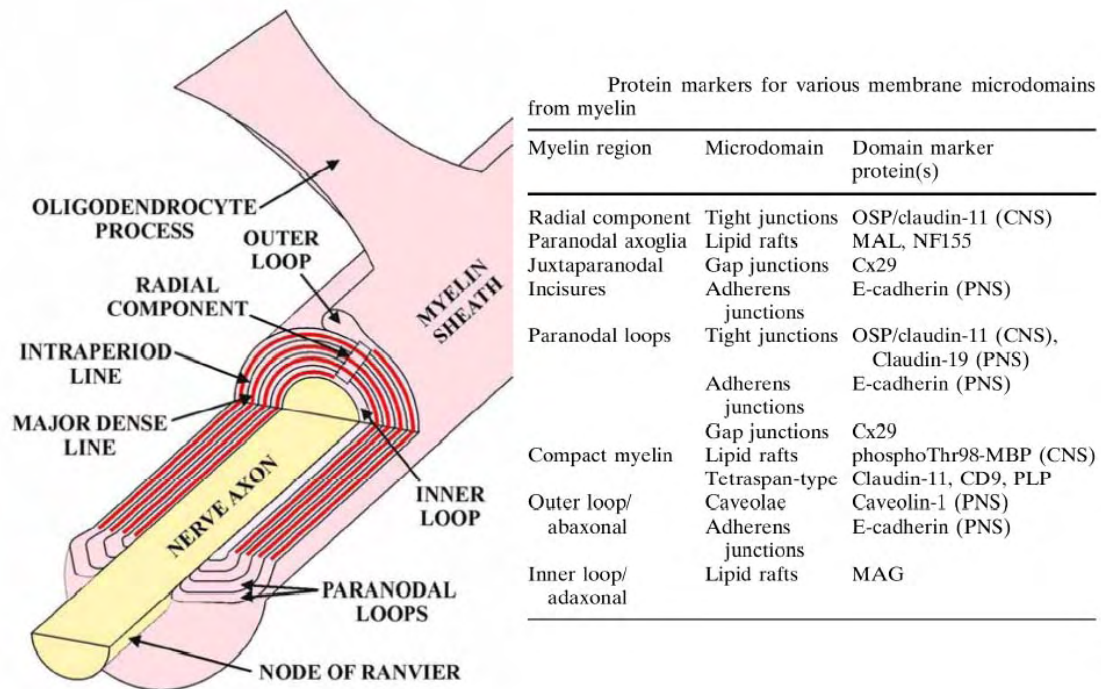


Figure 21. Schematic presentation of the intricate architecture of the myelin sheet and a table on the different microdomain within this structure. (Adapted from DeBruin L, Neurochem Res, 2007)

IgSF CAM members identified in the myelin sheet include MOG (myelin/OL glycoprotein) and MAG (myelin-associated glycoprotein), which are localised at the outer and periaxonal OL myelin membrane, respectively (figure 22). MOG is composed of one IgV-set domain, a transmembrane domain followed by a putative second transmembrane domain, a membrane linker and an intracellular domain. There are several splice variants of the protein with common extracellular but alternative cytoplasmic domains (Allamargot and Gardinier, 2007). MOG might play a role in regulating microtubule stability, in cell adhesion and/or in activating the complement pathway through direct binding with C1q (Johns and Bernard, 1999). MAG belongs to the IgSF subgroup Siglec (sialic-acid-binding Ig-like lectin) and is also called Siglec-4a (Quarles, 2007). It is composed of five Ig-like domains, a transmembrane domain and cytoplasmic domain. Two isoforms with alternative cytoplasmic domains have been

detected for this protein. MAG is selectively expressed in the periaxonal schwann cell and OL membranes of the myelin sheet. The localised expression and other data suggests that it is important for the initial OL contact with the axolemma as well as for wrapping the myelin sheet and maintaining this intricate contact site. Evidence suggests that MAG is involved in transmitting the myelination signal to the OL and it is also important for the maintenance of the axonal cytoskeleton and hence its integrity. The relative expression of MAG is higher in the CNS than the PNS, while in both cases it remains a minor component of the myelin sheet. MAG is phosphorylated and interacts with cytoplasmic signalling and adaptor proteins, including Fyn. It is heavily glycosylated and interacts with sialic acid residues on glycan structures, especially with $\alpha 2,3$ -linked sialic acid. The axonal receptors of MAG have not been clearly defined but it may bind to several components on the axolemma, including Neuregulin receptor 1 and 2, and gangliosides.

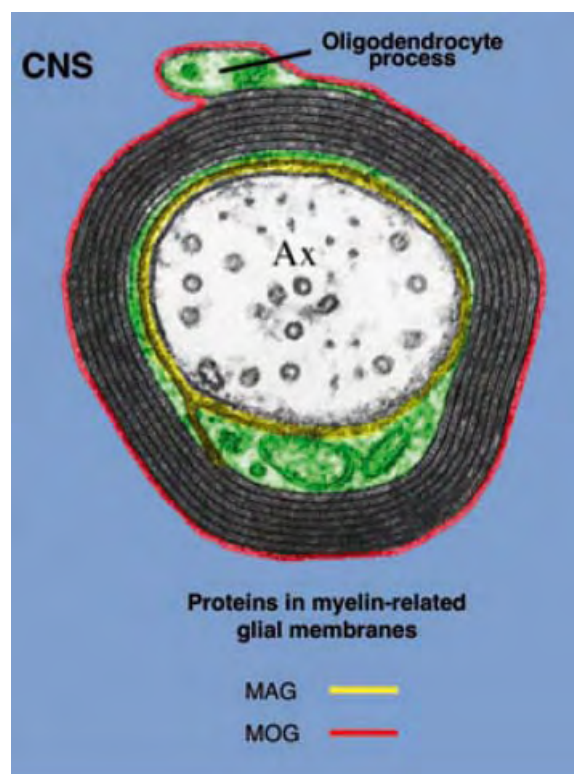


Figure 22. Electron micrographs of CNS myelin-sheaths color coded to show the locations of MAG (yellow) at the inner and MOG (red) at the outer layer of the myelin sheet. The cytoplasm of OL are highlighted in green. Proteolipid protein and MBP are the major proteins of CNS compact myelin (gray). (From Quarles RH, J Neurochem, 2007)

1.3.Gliomas and the role of IgCAMs

Since CAMs have important diverse roles in tissue architecture and homeostasis, as previously described, dysregulation of their expression is implicated in the development of many different tumours (Lafrenie et al., 2007). CAMs are involved in cancer cell proliferation, detachment, migration, survival, invasion and metastasis. As an example, downregulation of cadherins and upregulation of integrins are associated with increased tumour malignancy.

Brain tumours are generally classified according to the cell type that they most resemble histologically. However, often it is difficult to determine the cell type as many tumour cells show stem cell type characteristics. Despite the difficulties with the classification, 120 different types of brain tumours have been identified. Of these the most common ones are ependymomas, medulloblastomas in children, oligodendrogliomas, meningiomas in adults and different malignancy grades of astrocytomas (Collins, 2004). A general tumour grading scale from one to four has been introduced by the WHO, where grade I corresponds to low and grade IV to high tumour aggressivity. However, some tumour types have less than four different grades. Astrocytomas are the most common type of brain tumours and they can be divided into four malignancy grades; pilocytic astrocytomas (grade I), astrocytomas (grade II), anaplastic astrocytomas (grade III) and glioblastomas (grade IV) (Collins, 2002). Pilocytic astrocytomas are most commonly found in the cerebellum of children. They are generally non-aggressive and maintain their grade I phenotype for years or even decades. Histologically pilocytic astrocytomas show diverse cell phenotypes, from pilocytic, bipolar cell areas containing Rosenthal fibers (subtype of intermediate filament inclusions formed by aggregation of GFAP) to areas containing cells with eosinophilic granular body and clear cells. Grade II astrocytomas resemble astrocytes, have little nuclear atypia and undergo mitotic activity. Anaplastic astrocytoma cells are more pleomorphic, show distinct nuclear atypia and mitotic activity. Glioblastomas take on a wide range of morphologies and while some cells maintain astrocyte type characteristics others are very pleomorphic with giant forms. Glioblastomas include cells undergoing

mitotic activity, necrosis and endothelial proliferation. All the different grades of astrocytomas are motile, where pilocytic astrocytomas have a strong tendency to invade the subarachnoid space, the other grades largely invade the white matter and subsequently the grey matter regions (Subramanian et al., 2002). However, the rapid growth and higher invasion potential increase with the tumour grade. Glioblastomas occasionally also form metastasis and infiltrate the CSF. On the other hand, as for other types of brain tumours, primary brain astrocytomas very rarely form metastases.

While astrocytomas constitute about 60-75 %, oligodendrogliomas (OD) constitute about 5-20% of all gliomas. Pure OL tumours have a better prognosis than the equivalent grade of astrocytoma, however they still unconditionally lead to mortality. Patients with OD have 10-15 years while patients with anaplastic OD have 4-5 years median survival time. The current annual rate of OD incidents in Europe is 0.2 per 100.000 persons and hence it remains a reasonably rare brain tumour type (Van den Bent et al., 2008). However, there is neither clear diagnostic criteria nor reliable marker for ODs, which could lead to forged statistics. Indeed recent data suggests that ODs could be more common than once thought and that in the past many ODs were diagnosed as various types of astrocytomas. On the other hand, up until 20-30 years ago when it was demonstrated that ODs respond well to specific chemotherapy, there was no real need to segregate them from astrocytomas as the clinical treatment was the same. ODs are believed to originate from OLs due to their histological features even though this has not been clearly demonstrated and they do not express any of the characteristic OL antigens but in contrast may express GFAP (Collins, 2004). Overexpressing PDGF in nestin-positive OPCs *in vivo* generates brain tumours with an OD type histology, suggesting that ODs could be of OL origin (Dai et al., 2001). However, PDGF overexpression in GFAP-positive astrocytes gave the same result. ODs have been divided into two grades, namely oligodendroglioma (grade II) and anaplastic oligodendroglioma (grade III). About 77 % of ODs are the lower grade and 23 % anaplastic ODs (Engelhard et al., 2003). ODs are composed of monomorphic tumour cells with rounded nuclei and often artefactually swollen cytoplasm on paraffin sections, giving rise to the “fried egg” appearance. There is no or little mitotic activity, no microvascular proliferation and no necrosis. On the contrary, hallmarks of anaplastic ODs are mitotic activity, microvascular proliferation, and/or spontaneous necrosis. It has been shown that in 55 % of cases the primary location of the OD is in the frontal lobe, 47 % in the temporal lobe, 20% in the parietal lobe, 4 % in the occipital lobe, 3 % in the

cerebellum and 1 % in the spinal cord. ODs tend to invade the white matter regions and the subarachnoid space. They can also invade the subependymal space along the ventricles and in 5 % of cases seeding into the CSF can be detected. Clinical symptoms for OD does not reliably distinguish it from other brain tumours, these include seizures, headaches, mentality shifts, vertigo/nausea, visual complains and/or localized limb weakness. As previously mentioned, there are no reliable histological markers for OD and there are considerable interobserver variations in the diagnosis. The most robust objective marker for OD today is the allelic loss of the 1p and 19q loci. The loss of either loci occurs in about 75 % and the combined loss is observed in about 60-70 % of ODs. Furthermore, the absence of these loci are associated in a good medical outcome, as these patients generally respond well to the current OD chemotherapies. Other changes include methylation of p14ARF and overexpression of EGFR, PDGFR and PDGF in OD grade II. However, this highlights the desperate need for specific OD grade specific markers.

Since ODs are relatively rare compared to astrocytomas, the latter have been more extensively studied. Accordingly, very few studies been done on the role of IgCAMs in OD. However, several examples exist of altered IgCAM expression in astrocytomas. NCAM is downregulated in human astrocytomas and the expression level negatively correlates with tumour malignancy (Sasaki et al., 1998). The loss of NCAM could alter the cell-cell and cell-ECM adhesion of the tumour cells. Cavallaro U. *et al.* provide a hypothesis of how NCAM downregulation and consequent loss of cell-ECM adhesion could contribute to tumorigenicity (Cavallaro and Christofori, 2004). In normal conditions NCAM can bind FGFR and activates the receptor together with N-cadherin, which leads to a signalling cascade that ultimately induces β 1-integrin activation and cell-matrix adhesion. A defective cell-matrix adhesion due to a lack of β 1-integrin activation could lead to a passive release of tumour cells from their environment and consequent invasion. However, the exact outcome of NCAM downregulation on advanced tumorigenicity is still largely unknown.

CAR, a CTX family member described above, acts as a tumour suppressor in glioblastoma cells (Kim et al., 2003; Fuxe et al., 2003). CAR overexpression decreases invasion and intracerebral growth of glioblastoma cells. CAR is strongly expressed in the developing heart and nervous system, it is highly enriched in nerve growth cones and binds actin in in growth cone fractions isolated from mouse brain (Honda et al., 2000;

Huang et al., 2007). Furthermore, CAR may regulate cell migration through its interaction with tubulin, and more specifically microtubules (Fok et al., 2007). These findings suggest a role for CAR in processes requiring dynamic reorganization of the cytoskeleton such as neurite outgrowth, growth cone pathfinding, adhesion and motility. It is possible that CAR, as an adhesion molecule, exists as cis multimers *in vivo* and is able to crosslink microtubules at adhesion sites. It has been shown that microtubule-binding proteins, such as JAM-A, can inhibit cellular motility through microtubule stabilization (Bazzoni et al., 2005). It has been proposed that CAR may act similarly within a multiprotein complex to reduce microtubule dynamics to minimal basal activity level. Microtubules are known to play important roles in cell migration. For instance, during cell migration, microtubules are targeted to adhesion complexes where they promote cell migration through their contribution to the dissociation of adhesion sites from the substrate. Interestingly, JAM-A expression also inversely correlates with tumour aggressiveness (Naik et al., 2008).

In contrast to the two examples above, expression of Neuregulin and Contactin, two IgSF members, correlate with astrocytoma malignancy grade. Neuregulin (NRG) influences the migration of several cell types in the developing brain. NRG-1 mediates its effects via the activation of dimers of protein tyrosine kinase receptors, erbB2, erbB3, and erbB4. The protein exists as a membrane bound form in the nervous system, in glioma cells the extracellular portion can be proteolytically cleaved off, rendering a functionally active soluble protein (NRG-1 β) (Westphal et al., 1997). NRG-1 β enhances glioma cell (U251) motility, migration and survival (Ritch et al., 2003; Ritch et al., 2005). This is through an autocrine/paracrine stimulation leading to erbB2 activation and consequent induction of the focal adhesion complex, including β 1-integrin and FAK (focal adhesion kinase). In accordance, erbB2 is also overexpressed in human glioma autopsy samples. Contactin, of which the expression correlates with increased astrocytoma malignancy grade, does not increase glioma cell adhesion to the ECM or between cells nor proliferation, but enhances the repulsion between these cells (Eckerich et al., 2006). Interestingly, the current belief is that contactin is only expressed by neurons and astrocytes in the developing brain, which could support the hypothesis that tumour cells retrieve stem cell like characteristics.

1.4. GlialCAM, a novel cell adhesion molecule, is downregulated in human liver cancer

GlialCAM/hepaCAM/zig-1 is a member of the IgSF and is composed of two Ig-like domains, an N-terminal V-set domain followed by a C2-set, a transmembrane domain and an intracellular domain (figure 23). Two isoforms with variable intracellular domain lengths have been recently registered in the Swiss-prot database (Q14CZ8-1 and Q14CZ8-2). All the current work has focused on the longer 46 kDa isoform and the characterization and role of the 40 kDa isoform are still to be determined. The GlialCAM extracellular domain harbors both N- and O-linked carbohydrate moieties and the glycostructures contain several sialic acid residues (Gaudry et al., 2008; Chung et al., 2005). Its intracellular domain is relatively long for an IgCAM with low complexity

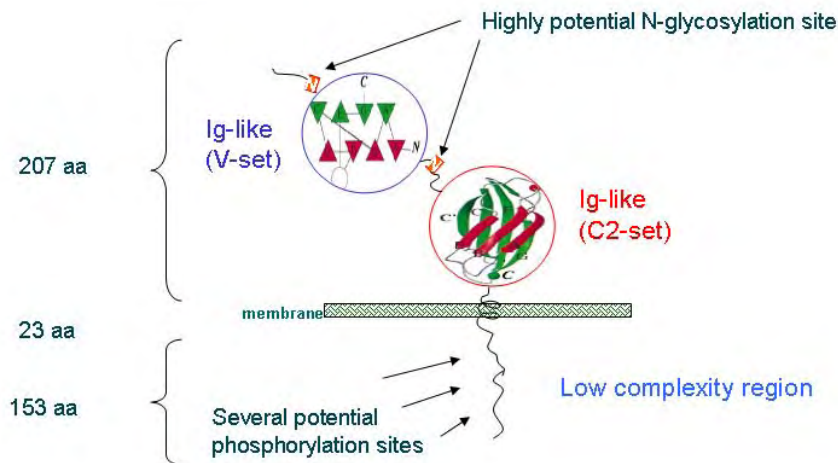


Figure 23. A schematic presentation of the GlialCAM structure. It contains an extracellular region with two Ig-like domains, an N-terminal V-set domain followed by a C2-set, and putative glycosylation sites, a transmembrane domain and a relatively long and low complexity intracellular domain with several putative serine/threonine and tyrosine phosphorylation sites.

and proline rich regions. Moh M et al. have indirectly demonstrated that the protein is phosphorylated when overexpressed in the MCF7 breast carcinoma cell line (Moh et al., 2005). GlialCAM shows sequence homology to CTX family members, including JAMs, ESAM, CAR and A33 antigen (figure 24). Both CAR and ESAM have a relatively long cytoplasmic tail like GlialCAM (153 amino acids), raising the possibility that GlialCAM

intracellular domain binds both signalling and adaptor proteins (Suzu et al., 2002). Other groups have shown that GlialCAM is expressed in human liver cells as well as in

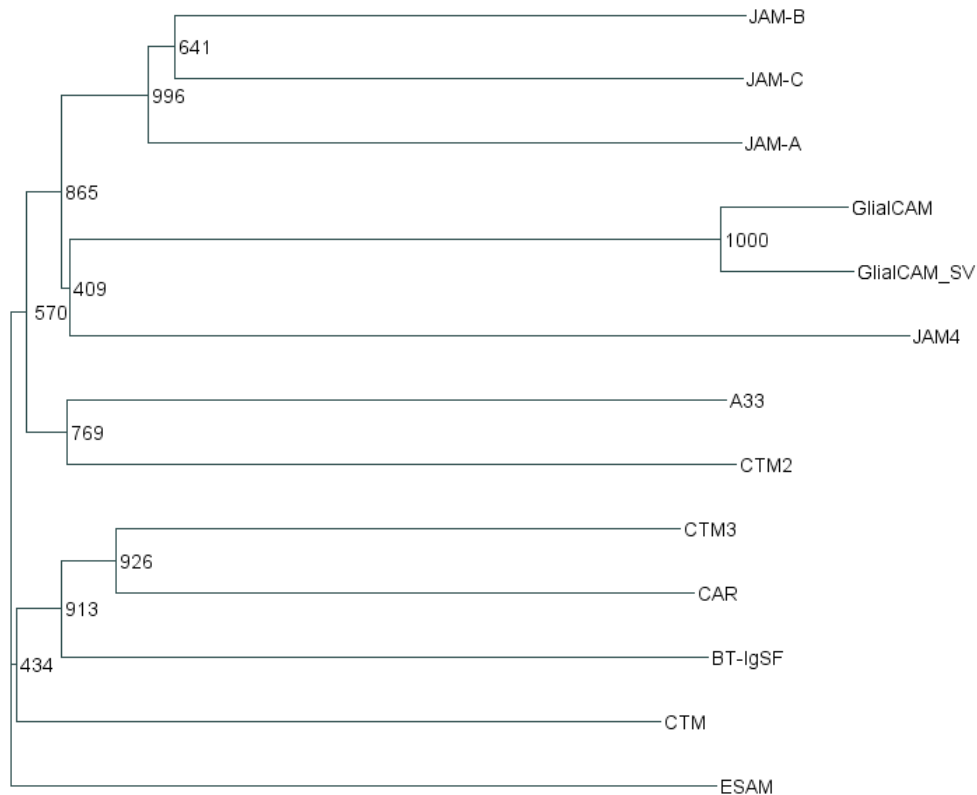


Figure 24. Human GlialCAM shows homology with CTX family members. An alignment was performed and a bootstrap tree was established using the ClustalW package. The tree was visualized using HyperTree. Accessions to the various sequences: GlialCAM isoform 1: Q14CZ89; GlialCAM isoform 2: Q14CZ8-2; CTM: NM_014312; CTM2: NM_182607; CTM3: NM_152538; BT-IgSF: NM_024769; CAR: NM_001338; A33: NM_005814; ESAM: NM_138961; JAM-A: NM_016946; JAM-B: NM_021219; JAM-C: NM_032801; JAM4: NM_001080444;.

dibutyryl cAMP stimulated primary rat Schwann cells and in newborn rat sciatic nerves (Chung et al., 2005; Spiegel et al., 2006). Furthermore, GlialCAM is downregulated in human hepatocellular carcinoma. It is mapped to the human chromosome 11q24, a loci which is frequently correlated with carcinogenesis (Koreth et al., 1999). Overexpression of GlialCAM in hepatic (Hep3B and HepG2) and breast tumour (MCF7) cell lines, where the protein is not otherwise expressed, induces increased cell motility on fibronectin and matrigel, delayed cell detachment on fibronectin coating and these functions are dependent on the cytoplasmic portion of the protein. Furthermore, overexpression of

GlialCAM in HepG2 cells reduces colony formation and inhibits cell growth. In the hepatic and breast tumour cell lines the cellular localisation of GlialCAM is cell density dependent, the protein localises to cell-cell junctions in confluent cell layers and to cell processes in dispersed cells. In polarised MCF7 cells GlialCAM is expressed at the basal and lateral surfaces and at the latter it colocalises with E-cadherin, however there is no direct interaction between these proteins. Moreover, GlialCAM colocalises with F-actin and F-actin depolymerisation leads to the disruption of the cellular distribution of the protein (Moh M, FASEB J, 2006 meeting abstract). Also, GlialCAM colocalises with Fyn and caveolin-1 in lipid rafts. Taken together, these data suggest a role of GlialCAM in the regulation of cell-matrix adhesion, cell growth and motility.

Aim of this work

As described above, CAMs are involved in many vital cellular functions and their deregulation often leads to pathologies, which include cancer, immune diseases and neurodegenerative disorders (Lafrenie et al., 2007; Weber et al., 2007; Poggi et al., 2007). There is increased data on targeting this protein family for therapeutic intervention in pathologies, with promising results (Schmidmaier and Baumann, 2008; Rychly and Nebe, 2006). Hence, we were interested in identifying and characterising novel CAMs for potential therapeutic applications.

Using a structure-based genome-mining approach (genome threader bioinformatic tool from Inpharmatica) targeting vascular endothelial growth factor receptor (VEGFR) and platelet derived growth factor receptor (PDGFR) Ig-like folds, we identified a sequence corresponding to a previously un-characterised single transmembrane domain containing protein with two Ig-like domains. The corresponding cDNA was cloned by exon assembly using a human brain genomic DNA library, the extracellular domain was expressed in a mammalian expression system and purified, with a good yield. When this work was initiated, there were no reports on the protein, however since then it has been published under the name hepaCAM and Zig-1, independently, and characterised in human liver, as described above (Chung et al., 2005; Spiegel et al., 2006).

The aim of our study was to determine the tissue and cellular expression profile of the protein, its molecular function and a possible physiological role. Based on the expression pattern we named the protein GlialCAM. Furthermore, we were interested in studying whether the extracellular domain could have a potential in therapeutic applications. The extracellular domains of several CAM proteins are solubilised through proteolytic cleavage *in vivo* and have physiological functions (Diestel et al., 2005; Westphal et al., 1997).

2.Results

The results obtained during this thesis are presented in three sections, where the first and the third sections are presented as papers. Preceding each section is an introduction covering the aims, tools, results and conclusion about the specific part.

2.1 GlialCAM, an Immunoglobulin-Like Cell Adhesion Molecule is Expressed in Glial Cells of the Central Nervous System

Favre-Kontula L. *, Rolland A. *, Bernasconi L., Karmirantzou M., Power C., Antonsson B. and Boschert U. *equal contribution
Glia. 2008 Apr 15;56(6):633-45.

2.2 GlialCAM enhances OPC migration *in vitro*, plays a role in CNS myelination and is downregulated in human gliomas

Favre-Kontula L., Rolland A., Alliod, C, Frossard M, Dauvillier J., Boschert U., Pouly S. and Antonsson B.
(Paper in preparation)

2.3 Detection and identification of plasma proteins that bind GlialCAM using ProteinChip arrays, SELDI-TOF MS, and nano-LC MS/MS.

Favre-Kontula L, Sattonnet-Roche P, Magnenat E, Proudfoot AE, Boschert U, Xenarios I, Vilbois F, Antonsson B.
Proteomics. 2008 Jan;8(2):378-88.

2.1. GlialCAM, an Immunoglobulin-Like Cell Adhesion Molecule is Expressed in Glial Cells of the Central Nervous System

Favre-Kontula L.*, Rolland A. *, Bernasconi L., Karmirantzou M., Power C.,
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Glia. 2008 Apr 15;56(6):633-45.

Aims and tools

Previous work from the institute has demonstrated that sGlialCAM has immunomodulatory role in a LPS induced mouse immune model. These findings gave us the incentive to study GlialCAM expression and function in further detail. Since the protein was completely novel when this work was started, the first goal was to determine in which tissues and cells GlialCAM is expressed. The work described in this section demonstrates GlialCAM expression in human, mouse and rat.

For this purpose we generated and characterised a rabbit polyclonal antibody against human GlialCAM extracellular domain. The purified polyclonal antibody also recognises mouse and rat variants of the protein. It was found to be suitable for Western blotting and immunocytochemistry, but not immunohistochemistry. Two commercial polyclonal anti-GlialCAM antibodies have also become available but the immunohistochemistry staining that was obtained with these antibodies on mouse tissues was not satisfactory either. Primers for qPCR were generated against GlialCAM cDNA. GlialCAM deficient mice were generated by replacing the GlialCAM gene with the β -galactosidase encoding gene.

Summary

We characterised GlialCAM expression by quantitative PCR, Western blot, immunocytochemistry and lacZ staining. We showed that GlialCAM is mainly expressed in the nervous system, in white matter tracts and ependymal cell lining of the ventricles, in ependymal cells composing the central canal of the spinal and in isolated sciatic nerve. We detected low level of GlialCAM expression in human liver, while none was detected in mouse. In purified rat primary CNS cells, GlialCAM is expressed in OLs and

astrocytes. In both cell types, the protein is expressed at the cell body as well as at the tip of and along processes extended by these cells. In OLs, GlialCAM expression co-localises with GAP-43, a marker for OL growth cone-like structures (Fox et al., 2006). Western blot analysis of mouse brain extracts showed two bands corresponding to GlialCAM, most probably two splice variants, that are differentially regulated during post-natal development. Furthermore, GlialCAM post-natal expression correlates with MBP expression.

GlialCAM expression in OL growth cone-like structures suggests that the protein is involved in OL cell-cell and/or cell-ECM adhesion. Furthermore, as mentioned above, the concomitant expression with MBP and strong expression in CNS white matter rich regions indicates that GlialCAM could play a role in CNS myelination. Strong GlialCAM expression in cultured astrocytes, compared to astrocyte *in vivo*, could suggest that the protein has a function in activated astrocytes. Cellular localisation also implies that it is involved in cell-cell and/ or cell-ECM adhesion, as in OLs. The ependyma function as a protective and metabolic barrier between the CNS and CSF and GlialCAM could be involved in cell-cell interactions found in tight junctions of these cells. The present results on GlialCAM expression pattern provide a basis for a panel of possible investigations on its function.

GlialCAM, an Immunoglobulin-Like Cell Adhesion Molecule is Expressed in Glial Cells of the Central Nervous System

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KEY WORDS

hepaCAM; ependymal cells; oligodendrocytes; astrocytes; myelination; GAP43

ABSTRACT

Using structure based genome mining targeting vascular endothelial and platelet derived growth factor immunoglobulin (Ig) like folds, we have identified a sequence corresponding to a single transmembrane protein with two Ig domains, which we cloned from a human brain cDNA library. The cDNA is identical to hepatocyte cell adhesion molecule (hepaCAM), which was originally described as a tumor suppressor gene in liver. Here, we show that the protein is predominantly expressed in the mouse and human nervous system. In liver, the expression is very low in humans, and is not detected in mice. To identify the central nervous system (CNS) regions and cell types expressing the protein, we performed a *LacZ* reporter gene assay on heterozygous mice in which one copy of the gene encoding the novel protein had been replaced with β -galactosidase. β -galactosidase expression was prominent in white matter tracts of the CNS. Furthermore, expression was detected in ependymal cells of the brain ventricular zones and the central canal of the spinal cord. Double labeling experiments showed expression mainly in CNPase positive oligodendrocytes (OL). Since the protein is predominantly expressed in the CNS glial cells, we named the molecule glial cell adhesion molecule (GlialCAM). A potential role for GlialCAM in myelination was supported by its up-regulation during postnatal mouse brain development, where it was concomitantly expressed with myelin basic protein (MBP). In addition, *in vitro*, GlialCAM was observed in various developmental stages of OL and in astrocytes in processes and at cell contact sites. In A2B5 positive OL, GlialCAM colocalizes with GAP43 in OL growth cone like structures. Overall, the data presented here indicate a potential function for GlialCAM in glial cell biology. © 2008 Wiley-Liss, Inc.

involved in many other physiological processes such as cellular motility, migration, proliferation, and differentiation (Rojas and Ahmed, 1999). CAMs are generally classified into four major families according to their structure: the integrins, the selectins, the cadherins, and the immunoglobulin (Ig) superfamily of adhesion molecules (IgCAMs), which constitute the largest repertoire in vertebrates (Barclay, 2003).

IgCAMs are particularly abundant in the nervous system (NS) where they are implicated in diverse stages of brain development and are important for brain morphology as well as for many general NS functions (Sakisaka and Takai, 2005). During early NS development, IgCAMs such as neural cell adhesion molecule (NCAM) or L1 are involved in neuronal migration, axon guidance, target recognition, and synapse formation. However, they also participate in the maintenance and function of neuronal networks in the adult (Dityatev et al., 2004; Maness and Schachner, 2007). The importance of IgCAMs in the central nervous system (CNS) has mainly been investigated in neurons but recent studies indicate that these proteins also play a role in migration, process guidance and target recognition in glial cells (Fox et al., 2006). Furthermore, several IgCAMs are involved in the formation and stability of the myelin sheath wrapping the axons through the interactions between axons and the myelinating cells of the central and peripheral NS, oligodendrocytes, and schwann cells, respectively. The envelopment of axons with myelin is essential for efficient nerve conduction, and thus for the proper functioning of the NS (Sherman and Brophy, 2005). Furthermore, various CAMs have been described to be important for the integrity of ependymal cells, which line the cavities of the CNS and make up the walls of the ventricles. These ciliated epithelial

INTRODUCTION

Cellular adhesion molecules (CAMs) are cell surface receptors mediating cell–cell and cell–extracellular matrix interactions. They are not only essential for the maintenance of organs and tissue structures, but are

This article contains supplementary material available via the Internet at <http://www.interscience.wiley.com/jpages/0894-1491/suppmat>.

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Received 12 September 2007; Accepted 21 December 2007

DOI 10.1002/glia.20640

Published online 21 February 2008 in Wiley InterScience (www.interscience.wiley.com).

cells function as a protective and metabolic barrier between the CNS and the cerebrospinal fluid (CSF) (Del Bigio, 1995).

Using a structure-based genome-mining approach (genome threader bioinformatic tool from Inpharmatica) targeting vascular endothelial growth factor (VEGF) and platelet derived growth factor (PDGF) Ig-like folds, we have identified a sequence corresponding to a single transmembrane domain containing protein with two Ig-like domains. The corresponding cDNA was cloned by exon assembly using a human brain genomic DNA library and was found to be identical to *hepatocyte cell adhesion molecule* (*hepaCAM*) (Chung et al., 2005). *HepaCAM*, located on human chromosome 11q24, is an IgCAM that was originally identified as a putative tumor suppressor gene in human liver. The *hepaCAM* cDNA coding sequence spans 7 exons and encodes a type I transmembrane glycoprotein of 416 amino acids which displays the typical structure of an IgCAM, such as JAMs (junctional adhesion molecules) and ESAM (endothelial cell-selective adhesion molecule). Through cell spreading, detachment, and wound healing assays it has been demonstrated that *hepaCAM* is a cell adhesion molecule involved in cell-matrix interactions (Moh et al., 2005). *hepaCAM* has also been suggested as a tumor suppressor gene, as it is strongly downregulated in hepatocellular carcinoma and shows antiproliferative properties *in vitro* (Chung et al., 2005). Recently, *hepaCAM* was identified in cDNA libraries obtained from dibutyryl cAMP stimulated primary rat Schwann cells and in newborn rat sciatic nerves, suggesting a potential role for *hepaCAM* in the NS and particularly, at early stages of axon-glia interactions and/or in myelination (Spiegel et al., 2006).

To better understand its potential function in the CNS, we examined the expression pattern of this novel IgCAM at the tissue, cellular, and subcellular level using real time PCR, western blot (WB), immunocytochemistry, and a LacZ-reporter gene assay. We show that the molecule is mainly expressed in the CNS in both humans and mice, with the strongest expression observed in glia-rich white matter regions of the brain, and in ependymal cells of the ventricular zones and the spinal cord central canal. No expression of *hepaCAM* could be detected in mouse liver and the levels detected in human liver were very low. In the brain, OL were identified as the major cell type expressing the molecule. Based on this expression profile, we named the molecule glial cell adhesion molecule (*GlialCAM*). The potential role of *GlialCAM* in myelination was supported by its increased expression during mouse brain development, correlating with changes in myelin basic protein (MBP) expression levels and by its detection *in vitro* in OL at various differentiation stages. Interestingly, in A2B5 and O4 positive OL, the protein colocalizes with GAP43 in OL growth cone-like structures. *GlialCAM* might have additional functions in astrocytes, since the protein is strongly detected at cell contact sites. Taken together, the data presented here indicates a potential function for *GlialCAM* in OL, astrocytes, and ependymal cell biology.

MATERIALS AND METHODS

cDNA Cloning, Protein Expression and Purification, Generation of Antibodies

Exons 1, 2, and 3 corresponding to amino acids 1–240 (extracellular domain) of *GlialCAM* were amplified from human brain DNA by PCR, based on Genbank sequence accession number FLJ25530 on chromosome 11 (NT_033899.6) using the following primers: exon1F, GCA GGC TTC GCC ACC ATG AAG AGA GAA AGG GGA GCC CTG TC; exon1R, TCA CCC CCT CCA GGG GGT CTG TCT GGA TCA GAA GAA; exon2F, TTC TTC TGA TCC AGA CAG ACC CCC TGG AGG GGG TGA; exon2R, GTG GCC TCG AAA TGG GCA CAT CTA CAG TAA GGT TGA; exon3F, CAA CCT TAC TGT AGA TGT GCC CAT TTC GAG GCC ACA; exon3R, GGA GCT TCT TCT GTA TAC GGT GAT CTT GAC AG. Exon 1F also contained the partial sequence of the Gateway attB1 site and a Kozak sequence. Exon 3R also contained an 11 base overlap with the 5' sequence of exon 4. The three exons were assembled by overlapping PCR and the full length PCR product was subcloned into a Gateway compatible version of the mammalian cell expression vector pEAK12D (Edge Biosystems) with a C-terminal 6-his tag sequence for expression of the extracellular domain in HEK293 cells.

Full length *GlialCAM* cDNA was amplified from a human brain cDNA library using PCR primers based on Genbank sequence accession number FLJ25530. The full length PCR product was also subcloned into a Gateway compatible version of pEAK12D (Edge Biosystems) for expression in HEK293 cells.

A polyclonal anti-*GlialCAM* antibody was generated by immunizing rabbits with 6-his tagged extracellular domain (amino acids 35–240) of *GlialCAM* purified by Ni²⁺ affinity chromatography from supernatants of transiently transfected HEK293 cells. The antibodies were captured on Protein A, affinity purified on 6-his-*GlialCAM* (1–207) immobilized on Affi-Gel[®] 10 gel (Bio-Rad, Hercules, CA), and finally passed over a column of immobilized 6-his-CD147 to remove potentially cross-reacting or nonspecific antibodies.

SYBR-Green Real Time PCR Analysis of Gene Expression

Total RNA was isolated from a panel of human and mouse tissues using the Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. For quantitative RT-PCR, we made cDNA from RNA preparations using random primers and Superscript II reverse transcriptase (Invitrogen). Quantitative PCR was then performed using a SYBR green PCR kit (Qiagen, Valencia, CA) and PCR products were detected using an ABI 7900 sequence detection system (Applied Biosystems, Foster City, CA). Human *GlialCAM* was amplified using primers 5'-TGCTCCTTTTGTCTACCT-TC-3' and 5'-CAC ACAG AAAGCAGAGC-3' and its expression was quantified with the housekeeping gene *glyceraldehyde 3-phos-*

phate dehydrogenase (*GAPDH*), which was amplified using primers 5'-CCACCCATGGCAAATTC-3' and 5'-GATGGGATTTCATTGATGACA-3'. Mouse *GlialCAM* was amplified using primers 5'-GGGAGAAGACCATCAACCT-3' and 5'-TGAGCTCCAGCACAGTGGTT-3' and its expression was quantified with the housekeeping gene β -actin and the following primers: 5'-AACCTAAGGCCAACCGTGA-3' and 5'-GCCTGGATGGCTACGTACATG-3'. The expression levels of the target genes were normalized to the internal housekeeping genes and analyzed using the SDS 2.2.2 software system (Applied Biosystems).

Western Blot Analysis

Human tissue lysates (Prosci Incorporated, Poway, CA) were supplied in SDS sample buffer containing 5% β -mercaptoethanol. Mouse tissue lysates were prepared as follows: specific organs from adult C57BL/six mice were removed after intracardial PBS perfusion and homogenized in 50 mM Tris pH 8 containing 150 mM NaCl, 0.02% NaN_3 , 0.1% SDS, 1% NP-40, 0.5% sodium deoxycholate, and an EDTA-free protease inhibitor cocktail (Roche, Basel, Switzerland) using a polytron. Large debris and unbroken cells were removed by centrifugation. The supernatant was collected and the protein concentration determined using a modified Bradford assay (Bio-Rad).

For WB analysis, 20 μg of tissue extracts were separated on a NuPAGE[®] 4–12% Bis-Tris gradient gel (Invitrogen) and transferred onto a 0.2 μm nitrocellulose membrane. Membranes were blocked with TBS containing 0.1% Tween-20 and 5% skimmed milk and incubated at 4°C overnight with primary antibody. After three washes, membranes were incubated with peroxidase-coupled secondary antibodies against rabbit and mouse IgG (Dako, Glostrup, Denmark). Bands were then visualized using an ECL detection system (GE Healthcare, Little Chalfont, United Kingdom). Mouse monoclonal anti-actin antibody (Chemicon, Temecula, CA) and anti-GAPDH antibody (Ambion, Austin, TX) were used as loading controls.

For the expression study during postnatal development, the total brain of C57BL/six mice was dissected at various time-points and treated as above. To quantify *GlialCAM* expression, four mice were analyzed per time point and protein bands were quantified by densitometric scanning ($\text{OD} \times \text{mm}^2$) using a GS-800 calibrated densitometer and related software (Bio-Rad). *GlialCAM* specific bands were then normalized to GAPDH band levels. To study the distribution of *GlialCAM* in mouse brain, we used an adult mouse brain blot containing 12 distinct brain areas of a Balb/c male (Chemicon). The blot was treated as described earlier.

Generation of *GlialCAM* Deficient Mice and *Lac-Z* Staining

GlialCAM deficient mice were generated using the Velocigene technology (Regeneron, Tarrytown, NY) as described previously (Valenzuela et al., 2003). Briefly, the

genomic sequence of *GlialCAM* was replaced by a cassette containing the coding sequence of β -galactosidase (*lac-Z*) and Lox flanked neomycin gene driven by a mammalian promoter (supplementary Fig. 1). For the *Lac-Z* reporter gene assay, in wild type and *GlialCAM* heterozygous mice, tissues were fixed by transcardial perfusion with PBS containing 4% paraformaldehyde. The tissues were removed, carefully dissected, frozen, and cut with a cryostat at a thickness varying from 10 to 40 μm . To measure β -galactosidase activity, sections were incubated with substrate solution for 12 h. The reaction was then stopped and tissue sections were counterstained with nuclear red.

To identify the cell types expressing *GlialCAM* *in vivo*, we performed double immunohistochemical staining on heterozygous tissue sections. Sections were incubated overnight at 4°C with the following primary antibodies (dilution 1:100 to 1:2,000): rabbit polyclonal anti- β -galactosidase (Europa Bioproducts, Cambridge, UK), anti-GFAP (glial fibrillary acidic protein) (Sigma-Aldrich, St. Louis, MO), and mouse monoclonal anti-CNPase (Covance, Berkeley). Sections were then incubated with the biotinylated anti-rabbit and anti-mouse IgG included in the ABC vector kits (Vector Laboratories, Burlingame, CA) that were used throughout the procedure according to the manufacturer's instructions. β -galactosidase staining was revealed with DAB (Black) and GFAP and CNPase stainings were revealed with streptavidin CyTM3 complex (Vector laboratories).

Primary Glial Cell Cultures

Glial cells were isolated from newborn Sprague-Dawley rat cortices essentially as described previously (McCarthy and de, 1980). Briefly, brain cortices were dissociated in HBSS (Invitrogen) containing 0.01% trypsin and 10 $\mu\text{g}/\text{mL}$ DNase I type IV (Sigma) for 10 min at 37°C. After centrifugation, cells were resuspended in DMEM containing 20% FCS, 4.5% glucose, 1% sodium pyruvate, 1% L-glutamine, and penicillin/streptomycin (Invitrogen), and filtered through a 70 μm mesh. The cells were grown on poly-D-Lysine flasks (BD Biosciences, Franklin Lakes, NJ) until confluent (approximately 7–9 days). At confluency, the flasks were rotated at 150 rpm for 1 h at 37°C. The detached microglia were collected and used in further experiments. Cell medium was replaced and the flasks rotated for 18–20 h. The detached OL precursor cells (OPCs) were filtered and resuspended in DMEM containing GlutaMAXTM I and sodium pyruvate, 0.1% BSA (fraction V) (Invitrogen), 30 nM Na-selenite (Sigma), 10 nM D-biotin (Sigma), 10 nM hydrocortisone (Sigma), 50 $\mu\text{g}/\text{mL}$ insulin (Sigma), 50 $\mu\text{g}/\text{mL}$ apotransferrin (Sigma), 10 ng/mL PDGF AA (Sigma), and 10 ng/mL bFGF (Abcys S.A., Paris, France). To induce OPC differentiation, cells were grown for 3 days in the presence of 30 nM T3 (Sigma), 20 ng/mL recombinant human CNTF (R&D systems, Minneapolis, MN), and 10 μM forskolin (Sigma). The remaining adherent astrocytes were detached using trypsin-EDTA (Invitrogen) and plated in DMEM containing 20% FCS.

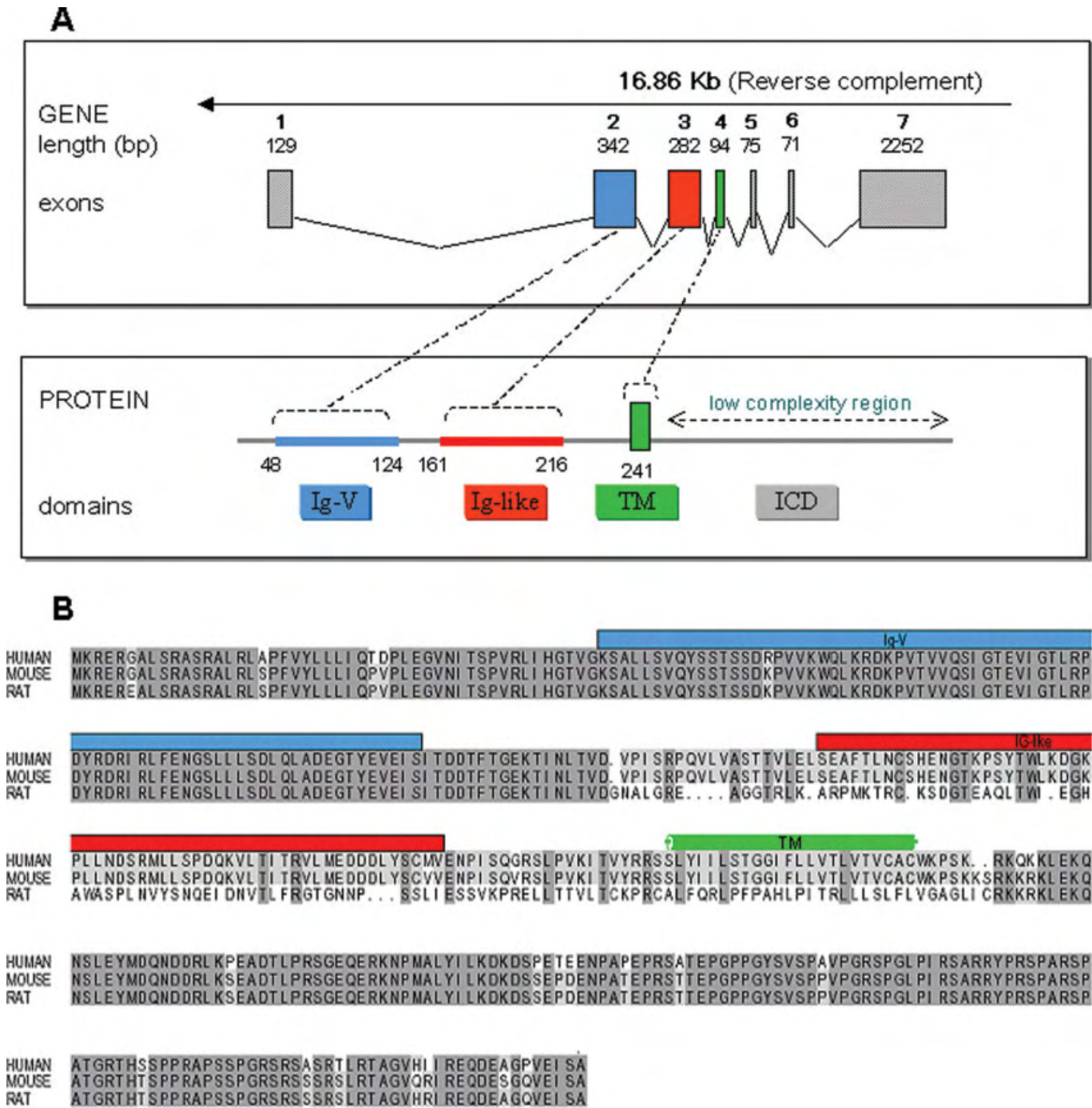


Fig. 1. GlialCAM structure and sequence alignments. **A:** *GlialCAM* gene organization and protein domain structure. Ig-V, immunoglobulin v-set domain; Ig-like, immunoglobulin like domain; TM, transmembrane domain; ICD, intracellular domain. **B:** Amino acid sequence alignments. Multiple alignments were computed with ClustalW, and the figure was generated with the program Alscript using amino acid sequences of human (ENST00000298251), mouse (ENSMUSP00000054105), and rat hepaCAM (ENSRNOP00000012248). Amino acids conserved in all three species are boxed in dark grey and those conserved in only two species are in light grey.

Immunocytochemistry

For immunocytochemical staining, glial cells were fixed with PBS containing 4% paraformaldehyde for 20 min. Cells were blocked with 10% goat serum (NGS) in PBS and incubated for 2 h at room temperature with

the following primary antibodies diluted in PBS containing 10% NGS and 1% saponin (Sigma): mouse anti-O4 (1:100 dilution; Chemicon), mouse anti-MBP (1:2,000; Chemicon), mouse anti-GAP-43 (1:2,000; Chemicon), mouse anti-GFAP (1:100; Chemicon), mouse anti-A2B5 (1:100; Chemicon), and rabbit anti-GlialCAM antibody

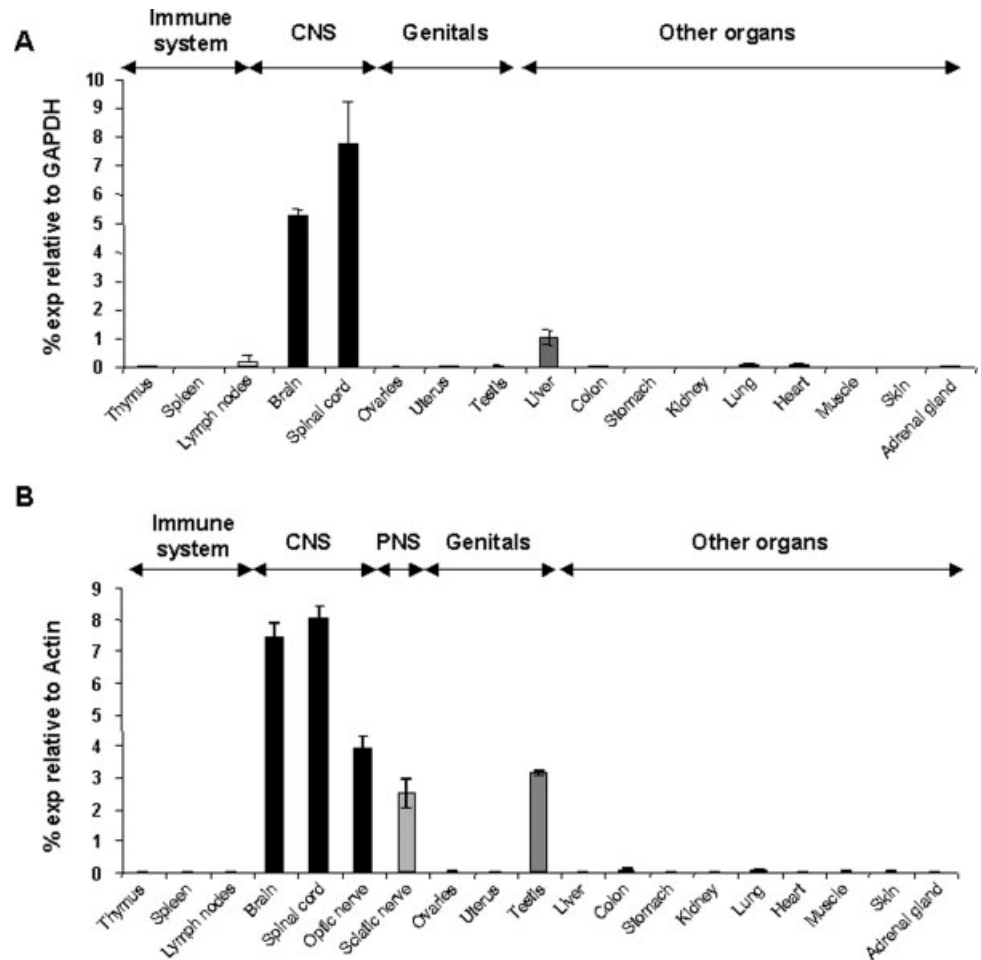


Fig. 2. *GlialCAM* gene expression profiling in human and mouse tissues. cDNA from human and mouse tissues were subjected to real-time quantitative PCR using specific primers for *GlialCAM*. *GlialCAM* expression was normalized to the internal housekeeping genes human *GAPDH* (A) and mouse actin (B). Human data are the mean of three experiments performed on the same cDNA set, while mouse data represents mean expression in three animals.

(1:200). Cells were then incubated for 1 h at room temperature with secondary antibodies: CyTM3-conjugated donkey anti-rabbit and CyTM2-conjugated donkey anti-mouse IgG (1:100 dilution; Jackson ImmunoResearch, West Grove, PA) after which the slides were mounted with Fluoromount (SouthernBiotech, Birmingham, AL) and visualized using a fluorescence microscope (Zeiss, Thornwood, NY).

RESULTS

GlialCAM Structure and Sequence

In Fig. 1A, we have illustrated the *GlialCAM* gene and protein domains. The gene contains 7 exons and encodes a 416 amino acid protein. The protein is composed of an extracellular region containing two Ig-like domains, one V-set and one C2 domain; a transmembrane region and a low complexity, proline-rich intracellular region (Moh et al., 2005). The structure of *GlialCAM* is similar to several members of the CTX (cortical thymocyte marker in *Xenopus*) gene family of adhesion molecules such as JAM and ESAM (Chung et al., 2005).

Homology searches of rat, mouse, and human amino acid sequences revealed that human *GlialCAM* is 94% identical to the mouse protein and 69% to the rat pro-

tein. The homology between mouse and rat *GlialCAM* protein is 73% (Fig. 1B). The identity between human and mouse amino acid sequence is even stronger within the extracellular domain of the protein where there is 99% sequence conservation. Therefore, the extracellular domain was chosen to produce *GlialCAM* rodent and human specific antibodies.

GlialCAM is Strongly Expressed in the Nervous System

We first used real-time quantitative RT-PCR to analyze *GlialCAM* expression in a variety of adult human and mouse tissues. In human tissues, we observed that *GlialCAM* was highly expressed in the CNS and was only expressed at low levels in liver (Fig. 2A). Interestingly, in mouse tissues (Fig. 2B), *GlialCAM* was also observed in the CNS but not in the liver. In mouse, *GlialCAM* mRNA was present in the sciatic nerve. *GlialCAM* was also detected in mouse but not human testis. None of the other human and mouse tissues tested expressed *GlialCAM* mRNA. Overall, these data demonstrate that the predominant site of *GlialCAM* expression in human and mouse is in the NS rather than in liver, where the protein has previously been characterized.

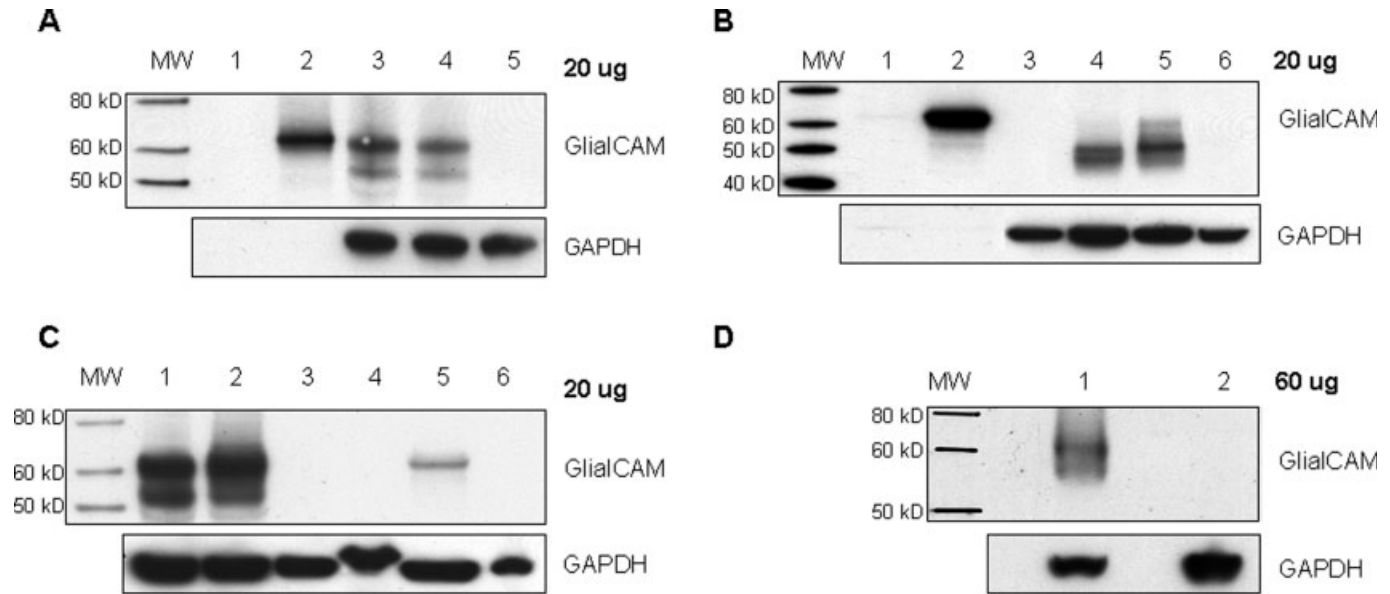


Fig. 3. GlialCAM is predominantly expressed in human and mouse CNS. **A:** The anti-GlialCAM antibody generated against the extracellular domain of the protein is specific. GlialCAM is detected by WB in HEK293 cells transfected with the full-length protein (lane 2), in wild-type (lane 3), and heterozygous mouse brain (lane 4) but not in control HEK293 cells (lane 1) and GlialCAM deficient mice brain (lane 5). **B:** In human adult tissues, GlialCAM is predominantly detected in brain and spinal cord. Lanes are as follows: 1, HEK293 cells transfected with

empty vector; 2, HEK293 cells transfected with GlialCAM; 3, testis; 4, brain; 5, spinal cord; 6, liver. **C:** In adult mouse GlialCAM is detected in brain, spinal cord, and sciatic nerve. Lanes are as follows: 1, brain; 2, spinal cord; 3, liver; 4, spleen; 5, sciatic nerve; 6, testis. **D:** When loading higher amounts of protein, GlialCAM is detected in human (lane 1) but not in mouse (lane 2) liver even after longer exposure. GAPDH was used as a loading control.

To confirm the expression pattern at the protein level, we generated a polyclonal antibody against the extracellular domain of GlialCAM. This antibody was affinity purified and its specificity was tested by WB (Fig. 3A) and immunocytochemistry (supplementary Fig. 2). This antibody recognizes both mouse and human GlialCAM. As shown in Fig. 3A, protein bands were observed in HEK293 cells transfected with human GlialCAM as well as in mouse brain. Moreover, no immunoreactivity was observed in brain lysate from GlialCAM deficient mice, thus confirming the specificity of the antibody. Interestingly, in wild type and GlialCAM heterozygous mouse brains, two specific bands were observed. Experimental data from our laboratory show that the two bands are glycosylated but that they do not converge into one single band after deglycosylation (data not shown). Hence, these bands may represent alternatively spliced forms of the molecule. This is supported by the UniProtKB/Swiss-Prot data base in which, two GlialCAM isoforms (entries: Q14CZ8-1 and Q14CZ8-2) differing by approximately 5.5 kDa in molecular weight are referenced (Ota et al., 2004).

Human and mouse tissues positive for GlialCAM mRNA were then analyzed by WB to determine the GlialCAM protein expression profile. In both human and mouse tissues, the protein expression level correlated with the mRNA expression profile, with the sole exception of mouse testis, where GlialCAM could not be detected. As shown in Fig. 3B, we observed strong expression of GlialCAM in human brain and spinal cord and low expression in human liver even after loading of higher amounts of protein and applying longer exposure times

(Fig. 3D). As observed in mouse, two GlialCAM protein bands were also seen in human brain. Mouse GlialCAM expression (Fig. 3C) was confirmed in brain, spinal cord, and to a lower extent in the sciatic nerve and optic nerve (data not shown). No GlialCAM expression could be detected in mouse liver even with higher amounts of protein loading (Fig. 3D), indicating that the protein is either not expressed or expressed at a very low level in this tissue. Thus, at the RNA and protein level, GlialCAM is strongly and predominantly expressed in the CNS.

GlialCAM is Expressed in Glial Cells of the Central Nervous System

To determine the expression of GlialCAM in the adult CNS, we first examined its expression by WB in anatomically and functionally distinct CNS areas of an adult Balb/c mouse. As previously demonstrated, on total mouse brain lysates, two GlialCAM bands were detected in all CNS regions tested. Strongest expression of both bands was seen in the cerebellum, entorhinal cortex, pons, medulla, and spinal cord (Fig. 4A). Lower GlialCAM expression levels were detected in the hippocampus, olfactory bulb, striatum, thalamus and midbrain.

To identify the major cell types expressing GlialCAM in the CNS, we performed a *Lac-Z* gene reporter assay on brain, spinal cord, and optic nerve sections from GlialCAM heterozygous mice expressing the *Lac-Z* gene. As shown in Fig. 4B, β -galactosidase activity was observed in the anterior (b) and posterior brain (c) of

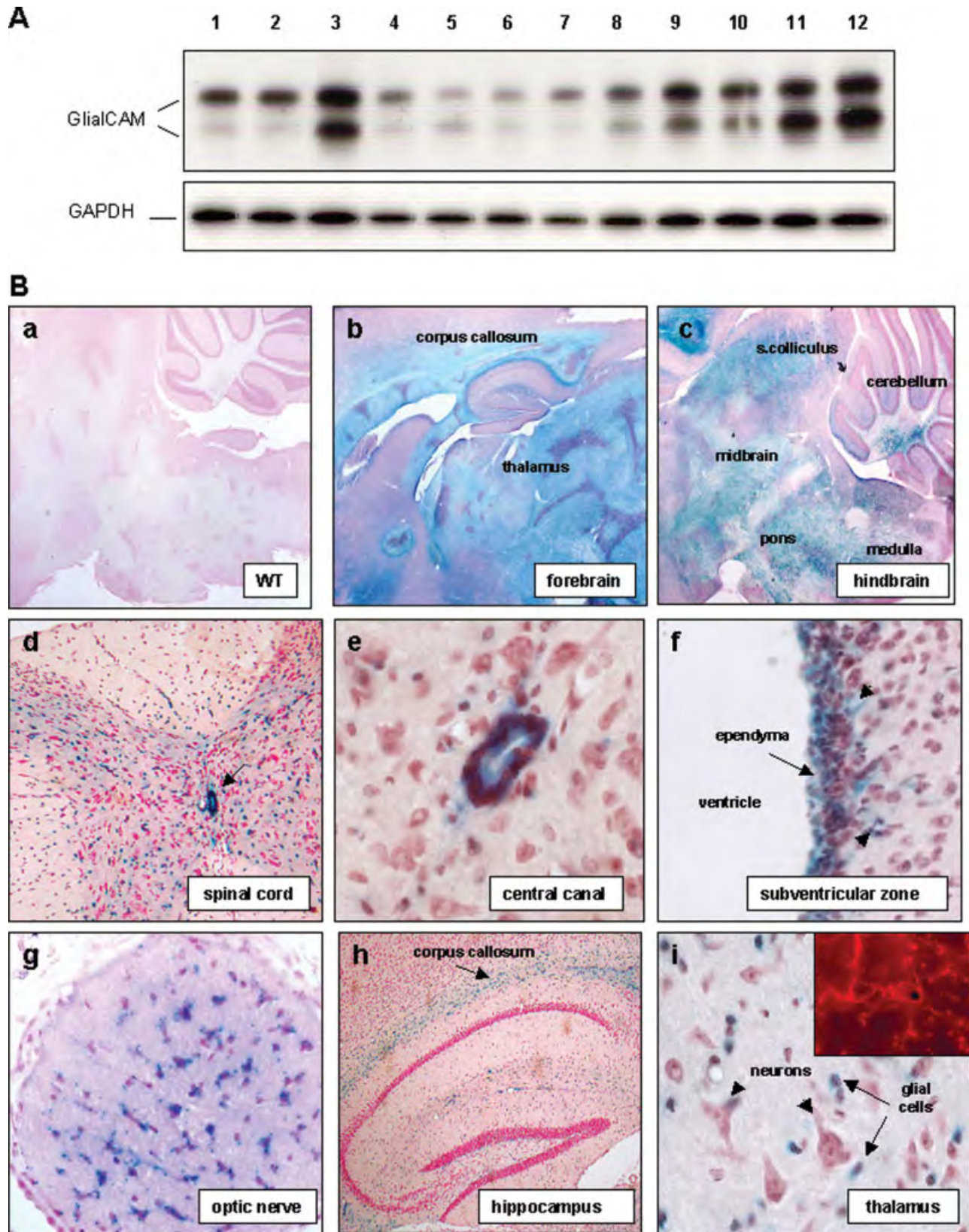


Fig. 4. GlialCAM distribution in mouse CNS. **A:** GlialCAM expression was tested by WB on various CNS regions of an adult Balb/c mouse. Strongest expression was detected in glia rich areas of brain and spinal cord. Lanes are as follows: 1, frontal cortex; 2, posterior cortex; 3, cerebellum; 4, hippocampus; 5, olfactory bulb; 6, striatum; 7, thalamus; 8, midbrain; 9, entorhinal cortex; 10, pons; 11, medulla; 12, spinal cord. **B:** *Lac-Z* reporter gene assay on GlialCAM heterozygous mouse brain, spinal cord, and optic nerve. Strong β -galactosidase activity was observed throughout the fore- (b) and hindbrain (c) but not in control WT littermates (a). In the spinal cord (d) GlialCAM was expressed in the grey and white matter and in the ependymal cells of the central canal (e). In addition, in the lateral ventricles (f) the ependymal (arrow) and subependymal layers (arrowhead) are stained. Within the optic nerve (g), the corpus callosum (h), and the thalamus (i), staining is seen in glial cells. Finally, using double stainings (i, insert) β -galactosidase activity (black, anti- β -galactosidase antibody) was seen (black, anti- β -galactosidase antibody) in CNPase positive OL (red fluorescence).

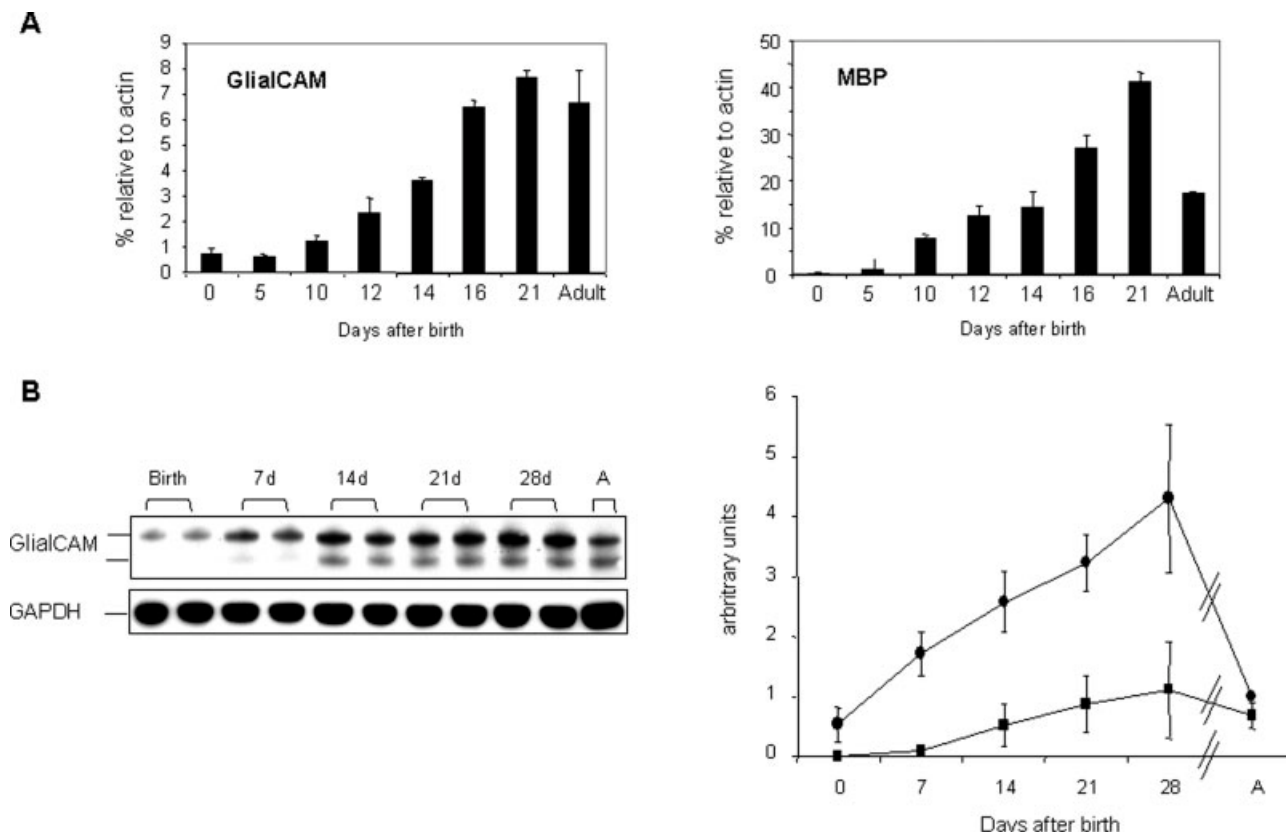


Fig. 5. GlialCAM expression during postnatal brain development. **A:** GlialCAM (left) and MBP (right) mRNA expression in mouse cerebellum between birth and adult age. Results are expressed as a percentage of expression normalized to actin expression and represent the mean expression in three animals. **B:** Left: WB analysis of GlialCAM expression in total mouse brain lysates. An upper band (63 kDa) is already detectable at birth. Its intensity increases until 4 weeks of age and

then decreases in adult mice. A second lower MW band appears 7 days after birth and increases in intensity until adult. Two mice are shown per time point. Right: The intensity of the GlialCAM bands was determined in four mice by densitometric scanning, and normalized with respect to the GAPDH loading control band. Arbitrary unit: GlialCAM to GAPDH ratio in adult mice is equal to one.

GlialCAM heterozygous mice while no staining was seen in WT littermates (a). Interestingly, glia rich areas like the corpus callosum, the pons, and the medulla as well as the white matter of the cerebellum were strongly positive. In the spinal cord, GlialCAM expression was localized to small cell bodies in the grey and white matter (d) and strong expression was also detected in ependymal cells of the central canal (d arrow, e magnification). Ependymal cells were also stained in the ventricular zones of the brain (f, arrow) and in the subventricular zone of the lateral ventricles, additional cell types might express the protein (f, arrowhead). Double labeling experiments need to be performed to reveal the identity of these cells. At a higher magnification, β -galactosidase activity was mainly observed in small cells resembling glial cells within the optic nerve (g), corpus callosum (h), and thalamus (i). In the thalamus, as well as in other brain regions (not shown), neurons (i, arrowheads) did not express GlialCAM. Finally, β -galactosidase activity was tested in many other organs from GlialCAM heterozygous mice including liver, and no expression was detected (data not shown).

The strong β -galactosidase reactivity observed in white matter regions of the brain suggests that glial

cells might express GlialCAM. To identify the glial cell types expressing GlialCAM, we performed double immunostaining using the glial markers: GFAP (astrocytes), CNPase (OL), and isolectin B4 (microglia cells) combined with anti- β -galactosidase immunostaining. As shown in Fig. 4B (i, insert), we observed β -galactosidase immunoreactivity in the cell body of CNPase positive OL. However, we also detected cells expressing β -galactosidase that were negative for CNPase labeling. Using GFAP/ β -galactosidase double labeling, we were unable to confirm if some of these additional lacZ positive cells were astrocytes. The complex GFAP staining pattern made the analysis difficult. No isolectin B4/ β -galactosidase positive microglia cells were detected in the different brain regions analyzed. We are currently characterizing various GlialCAM antibodies for their potential use in immunohistochemistry to perform double labeling experiments.

GlialCAM is Upregulated During Postnatal Brain Development

The expression of GlialCAM was then studied by quantitative RT-PCR in mice cerebella isolated at vari-

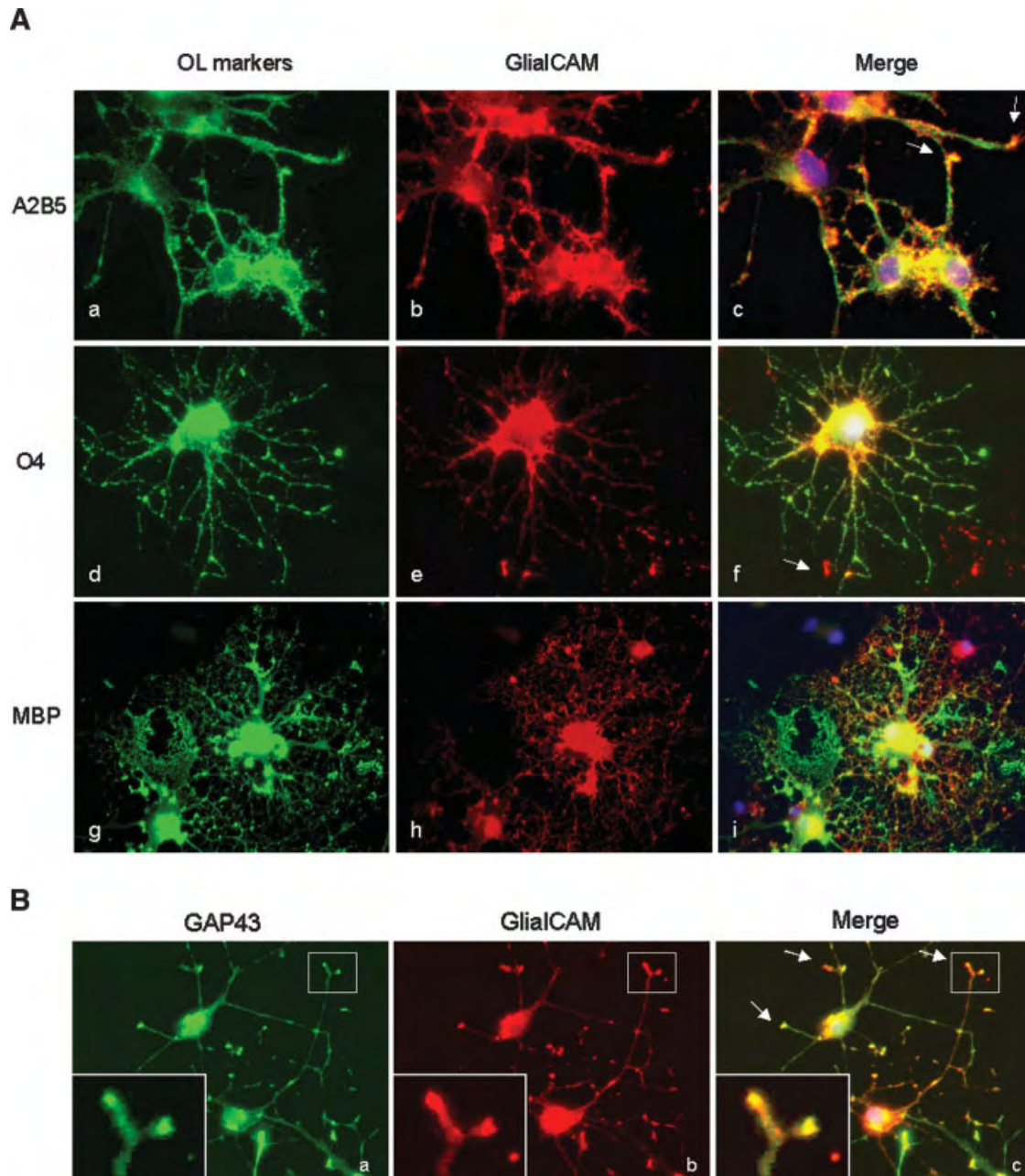


Fig. 6. Cellular and subcellular localization of GlialCAM in primary OL at different maturation stages. **A:** Immunocytochemistry using OL differentiation markers A2B5, O4, and MBP on purified OL shows that GlialCAM is detected in all three differentiation stages. Note that GlialCAM is expressed in the protruding tips (C) of early stage OLs. **B:** GlialCAM immunostaining colocalized with GAP43 (white arrows) in OPCs, indicating localization in growth cone-like structures. Higher magnification inset is seen in the left corner.

ous stages of postnatal development (P0–P21) and in adults. As shown in Fig. 5A, low amounts of mRNA were already detectable at birth (P0) and a steady increase of expression was seen from postnatal stage P10 to P21. Interestingly, the profile of GlialCAM expression follows that of MBP. GlialCAM protein expression was then studied by WB and the two specific bands were quantified individually by densitometry and normalized to GAPDH levels. As shown in Fig. 5B, the upper 63 kDa GlialCAM band is already detectable at

birth, increasing in intensity during postnatal development until P21 and decreasing slightly in adult animals. Starting from day 7 after birth, a second lower MW GlialCAM band (≈ 55 kDa) appears which also increases in the later postnatal stages, but did not show a significant decrease in adult brain. In summary, our results demonstrate that GlialCAM mRNA and protein are upregulated during postnatal CNS development. Its coordinated expression with MBP suggests that GlialCAM might be expressed in various stages of the OL lineage.

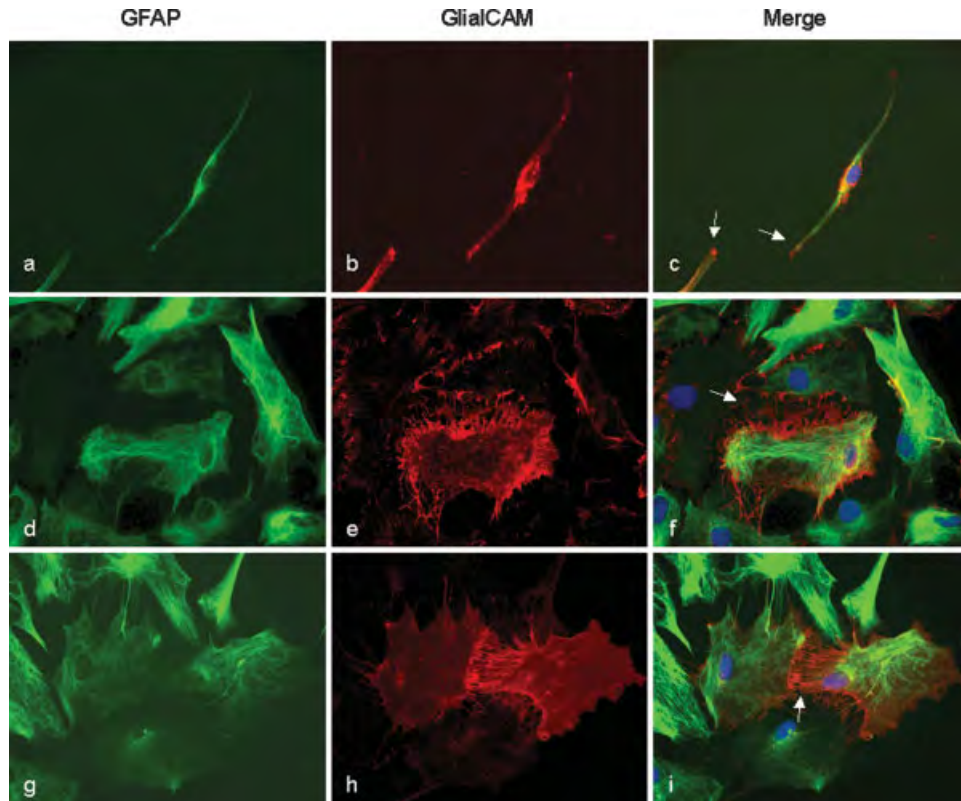


Fig. 7. Expression of GlialCAM in primary rat astrocyte cultures. Immunocytochemistry on primary rat astrocyte cultures shows that in bipolar astrocytes expressing GFAP, GlialCAM is detected on the cell body and at the tip of the processes (white arrows, c). In star shaped astrocytes, GlialCAM is seen on the cell body and on the processes connecting cells (white arrows, f and i).

GlialCAM is Expressed at Different Stages of Oligodendrocyte Differentiation *In Vitro*

Given the observed expression of GlialCAM in CNPase positive OL *in vivo*, we decided to analyze its expression in these cells *in vitro*. The differentiation of OL *in vitro* is a well-defined process associated with the expression of differentiation stage-specific myelin proteins/lipids and with characteristic changes in cell morphology (Baumann and Pham-Dinh, 2001). We used OL markers to identify differentiation stages (Fig. 6A): A2B5 (tetrasialogangliosides) for OPCs (a), O4 (sulfatide/seminolipids) for transient pro- and mature OL (d) and MBP for mature OL (g). GlialCAM expression was studied using double immunocytochemistry on purified rat A2B5⁺ OPCs and O4⁺ and MBP⁺ immature and mature OL. As shown in Fig. 6A(b,e,h), GlialCAM is expressed in all the three stages of OL differentiation and was observed in the cell body as well as in the processes. Interestingly, in A2B5 and O4 positive cells, expression could also be seen at the tips of some processes (arrows c, f).

To test whether the punctuate staining of GlialCAM localizes to OL growth cone-like structures, we double labeled the cells with GAP-43, a regulator of cytoskeletal organization, that was shown to be expressed in OL growth cones (Deloulme et al., 1990; Fox et al., 2006; Strittmatter et al., 1995). As seen in Fig. 6B GlialCAM

colocalizes with GAP43, confirming its localization to the growth cones and suggesting a function for GlialCAM in OL process extension, motility and guidance.

GlialCAM is Expressed in Primary Astrocytes *In Vitro*

The lacZ staining performed on GlialCAM heterozygous mice suggested that additional glial subtypes might express the protein. To determine if astrocytes or microglia express GlialCAM, we performed immunofluorescence staining on purified rat astrocytes and microglia cells. GlialCAM expression was not detected in microglia (data not shown). In contrast, we observed a strong expression in GFAP positive astrocytes (see Fig. 7). Bipolar astrocytes expressed GlialCAM in the cell body and at the tips of the processes (Fig. 7c). In star-shaped astrocytes grown at higher density, GlialCAM was strongly expressed on the numerous processes at cell contact sites (Fig. 7f,i), suggesting a role for the molecule in astrocyte/astrocyte interactions. This strong expression of GlialCAM in primary astrocytes might also reflect an astrocyte activation state *in vitro*. Further experiments are needed to determine if GlialCAM is expressed in resting astrocytes *in vivo* in normal

mouse brain, or if it is upregulated in models of reactive gliosis.

DISCUSSION

In the present study, we provide evidence that *GlialCAM*, previously identified as a putative tumor suppressor gene in human liver, is strongly and predominantly expressed in the human and mouse NS. In murine CNS, we demonstrate strong expression in various CNS regions like the cerebellum, the entorhinal cortex, the pons, the medulla, and the spinal cord. OL were identified as the major cell type expressing *GlialCAM* *in vivo*. In addition, we also observed strong expression of *GlialCAM* in ependymal cells along the brain ventricles and the central canal of the spinal cord. A potential role of *GlialCAM* in myelination was further supported by its temporal upregulation during postnatal mouse brain development, where it is coordinately expressed with MBP. *In vitro*, *GlialCAM* protein expression was detected in three developmental stages of the OL lineage. In A2B5 and O4 positive OL, *GlialCAM* colocalizes with GAP43 in OL growth cone-like processes. *GlialCAM* was also observed in primary astrocytes. Taken together, the present data indicate a potential function for *GlialCAM* in OL, astrocyte, and ependymal cell biology.

The *GlialCAM* sequence shares similarities with members of the CTX (cortical thymocyte marker in *Xenopus*) family of adhesion molecules including JAM-3 and ESAM (Chung et al., 2005). These members have been identified as trans-membrane components of tight junctions (TJ) in a wide range of tissues in endothelial and/or epithelial cells (Arrate et al., 2001; Eguchi et al., 2005; Hirata et al., 2001). Although the precise physiological role of *GlialCAM* remains to be elucidated, its significant homology to the CTX family together with its overall expression pattern might indicate a potential function in the formation of TJ like structures in OL, astrocytes and in the CNS ependyma.

The CNS ependyma is made of a single layer of ciliated epithelial cells named ependymal cells, which function as a protective and metabolic barrier between the CNS and CSF (Del Bigio, 1995). Ependymal cells are interconnected via numerous gap junctions, and some ependyma also express TJ associated proteins such as occludins and ZO-1 (Lippoldt et al., 2000; Petrov et al., 1994). Future studies using *GlialCAM* deficient mice might help to elucidate the potential role of *GlialCAM* in the CSF/CNS barrier. In addition to the ependymal cell expression, *GlialCAM* positive cells were detected in the sub-ependymal layer of the lateral ventricles. The sub-ventricular zone, the adult derivative of the embryonic forebrain germinal zones, is a site of neurogenesis and gliogenesis in the adult mammalian brain (Menn et al., 2006; varez-Buylla and Garcia-Verdugo, 2002). Further studies are needed to unravel a potential function of *GlialCAM* in these cellular functions.

Astrocytes do not form regular TJ in normal conditions but were shown to express the TJ associated pro-

tein claudin-1 under proinflammatory conditions, suggesting the formation of rudimentary TJs at astrocyte/astrocyte contacts during reactive astrogliosis (Duffy et al., 2000). We were unable to detect clear *GlialCAM* expression on astrocytes *in vivo* by *LacZ* staining, although the pattern of expression suggested that some of the positive cells might be astrocytes. In contrast, *in vitro* immunocytochemistry studies clearly showed strong *GlialCAM* expression in primary rat astrocytes expressing the reactive astrocyte marker, GFAP. The strong expression *in vitro* might be explained by the fact that cultured astrocytes are in a different activation state than resting astrocytes *in vivo*. Further studies are needed to investigate the role of *GlialCAM* in astrocytes, like its behavior on proinflammatory challenge *in vitro* or during reactive astrogliosis *in vivo*. In a confluent astrocyte layer, the protein is particularly localized at cell-cell contact sites. This observation is in accordance with a recent study showing that in a confluent culture of stably transfected MCF7 cells (human breast carcinoma cells), *GlialCAM* was recruited to the sites of cell-cell attachment (Moh et al., 2005). However, in low-density astrocyte cultures, we observe that the protein is localized at the tip of cell processes. This expression is in line with the observation by Moh et al., that at a lower cell density, *GlialCAM* is localized to punctuate structures in the perinuclear membrane, cytoplasm, and at the tip of the cell surface protrusions of stably transfected MCF7 cells (Moh et al., 2005). The expression of *GlialCAM* in the processes of bipolar astrocytes also suggests a role for this molecule in process extension or differentiation. Together, these observations suggest a function for *GlialCAM* in astrocyte/astrocyte and astrocyte/extracellular matrix interactions as well as in astrocyte growth and migration.

Unlike astrocytes, OL express TJ like structures under normal conditions. OSP/claudin-11, a major component of CNS myelin, is known to form TJ strands within myelin sheaths and has been proposed to have a structural role in myelin formation and maintenance (Gow et al., 1999; Morita et al., 1999). Based on our observation of *GlialCAM* expression in white matter regions of the adult brain and in primary, MBP-positive mature OL *in vitro*, *GlialCAM* might play a similar role in maintaining the myelin structure. In addition, the postnatal expression profile of *GlialCAM*, which correlates with MBP levels and myelin formation, supports a role for *GlialCAM* in myelination and/or in the maintenance of the myelin sheaths.

We have also shown that in cultured OL, *GlialCAM* is expressed in early differentiation stages and colocalizes with GAP-43 to the tips of the processes. GAP-43 is a regulator of cytoskeletal organization known to mediate neuronal growth cone navigation (Meiri et al., 1986). Growth cones are found at the tip of growing axons and have the ability to respond to extracellular directional cues and to mediate target recognition (Strittmatter et al., 1995). Little is known about growth cones in OL, but recent data suggest the existence of such structures

expressing GAP-43 in the processes of postmigratory premyelinating OL (Fox et al., 2006). Interestingly, processes extended by postmigratory OL ultimately participate in the formation of the CNS myelin sheath (Richter-Landsberg, 2001; Rumsby et al., 2003). Thus, OL growth cones are probably involved in OL migration from the germinal zones to their final destination, as well as in the search for axons through the recognition of extracellular cues. Taken together, the cellular localization of GlialCAM in growth cone like structures as well as the temporal regulation of the protein during development suggests a role for GlialCAM in OL migration, guidance and myelination.

In summary, our data demonstrate that GlialCAM is a two Ig domain containing adhesion molecule of CNS glia. GlialCAM expression in OL, astrocytes, and ependymal cells suggests a multifunctional role for this protein in the formation and maintenance of the myelin sheath, as well as in astrocyte and CSF/CNS barrier function. We are currently investigating if GlialCAM deficient mice show any defects with respect to these structures and functions.

ACKNOWLEDGMENTS

We would like to thank Christopher Phelps, Richard Fagan, and Andrew Davids from Inpharmatica Co., who initially identified the GlialCAM gene by structure based genome mining, Mariastella de Tiani for cDNA cloning and generation of expression constructs, Patricia de Lys and Alexandra Michel for their help in the characterization of the first GlialCAM antibodies, Maria Chiara Magnone for the initial *LacZ*-gene-reporter assay setup, and Dr. Laurent Galibert for helpful discussions.

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2.2. GlialCAM enhances OPC migration *in vitro*, plays a role in CNS myelination and is downregulated in human gliomas

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Aims and tools

Following the results that GlialCAM is mainly expressed in CNS glia, we decided to study its function in OLs and astrocytes in more detail. Structural analysis demonstrated that GlialCAM shows homology with CTX family members, which function as adhesion molecules. CTX proteins form homo- and heterotypic cis and trans interactions, therefore our first goal was to study possible GlialCAM homotopic interactions. Furthermore, these proteins do not only have adhesive, but also signalling functions, and given that GlialCAM has a longer intracellular domain than the CTX family members and the results by Moh M et al. (Moh et al., 2005) suggesting that GlialCAM is phosphorylated after ectopic expression in breast tumour cells, we were interested to study whether the protein is phosphorylated.

GlialCAM expression in cell processes suggests that it is involved in OL adhesive functions. Since OL growth cone-like structures, are important for OPC directed motility, we decided to study whether sGlialCAM would interfere with OPC migration. Furthermore, expression in white matter rich regions suggests a role of GlialCAM in myelination, encouraging us to study possible myelination abnormalities in GlialCAM deficient mice. Finally, Moh et al. have demonstrated that GlialCAM is downregulated in hepatocellular carcinoma (Chung et al., 2005), which prompted us to study GlialCAM expression in oligodendroglioma and astrocytoma.

In order to study GlialCAM functions mentioned above, we set-up cis and trans adhesion assays. Phosphorylation was studied using metabolic labelling with ^{33}P as well as *in vitro* kinase assay using [^{33}P]-ATP. Proliferation was studied by DNA labelling with [^3H]-thymidine. Classical molecular biology, biochemistry and cell biology techniques, such as plasmid cloning, immunoprecipitation and cell transfection were used. Furthermore, we developed a functional assay to study OPC migration.

Summary

We demonstrate for the first time that GlialCAM is glycosylated in mouse brain and that deglycosylation of the protein leads to its rapid degradation, that it is phosphorylated in cultured astrocytes and that ERK2 and ASK1 phosphorylates GlialCAM intracellular domain *in vitro*. Furthermore, we do not detect GlialCAM cis-dimerisation, while the protein forms trans-interactions. Treating OPCs with sGlialCAM enhances their migration, presumably due to reduced GlialCAM mediated contact between OPCs. This is supported by the fact that GlialCAM deficient OPCs show enhanced migration compared to WT controls. GlialCAM deficient mice show reduced levels of myelin associated proteins, MBP and CNPase, and vacuolisation within white matter tracts. Finally, the protein is downregulated in high grade oligodendrogliomas and both low and high grade astrocytomas, presumably through DNA hypermethylation. Taken together, these results suggest a role of GlialCAM as both an adhesive and signalling molecule in OLs and astrocytes. It seems to play a functional role both in early stage OPCs as well as mature myelinating OLs. Furthermore, it could act as a tumour suppressor gene in oligodendroglioma and astrocytoma.

2.2.1. Results

2.2.1.1. GlialCAM is glycosylated in mouse brain and exists as two isoforms

Western blot analysis of GlialCAM expression in mouse brain showed two protein bands that were differentially regulated during development. In order to determine whether the two bands represent differentially glycosylated forms of the protein, we treated adult mouse brain lysate with a mixture of deglycosylating enzymes, including sialidase, PNGase F, O-glycosidase, β -galactosidase and glucosaminidase for 0, 4, 8 and 24 h at 37 °C. The samples were analysed by Western blot and unexpectedly the GlialCAM band intensity decreased in a linear manner with the time of treatment with glycosidases, while incubation without glycosidases did not alter the GlialCAM band intensity (Figure 25 a). No detectable increase in aggregation (GlialCAM signal at the top of the gel) was detected on the Western blot of the treated samples and the aggregated material could not have been lost in the tubes as SDS-PAGE sample buffer was added directly into the treated samples in the eppendorff tubes. The decreased GlialCAM signal was not due to loss of the antibody epitope, since deglycosylation of sGlialCAM under the same conditions rendered a sharp immunoreactive band corresponding to the calculated molecular weight of sGlialCAM (figure 25b). These results indicate that following deglycosylation, GlialCAM is quickly degraded in mouse brain lysate, suggesting an importance of glycosylation for the stability of the protein. In the samples treated with deglycosylation enzymes for 4 h and 8 h, two weak bands were detected on the Western blot. The two bands could also be detected in the sample treated for 24 h after a long exposure time of the Western blot (figure 25c). The position of these bands corresponds to a lower molecular weight (Mw) on the gel compared to the bands in the untreated sample. Since the two bands did not converge into one after deglycosylation, this suggests that there are presumably two splice variants of GlialCAM in mouse brain. This is supported by a recent UniProtKB/Swiss-Prot data base entry in which, two GlialCAM splice-variants (entries: Q14CZ8-1 and Q14CZ8-2) differing by approximately 5.5kDa in molecular weight are described (Ota et al., 2004).

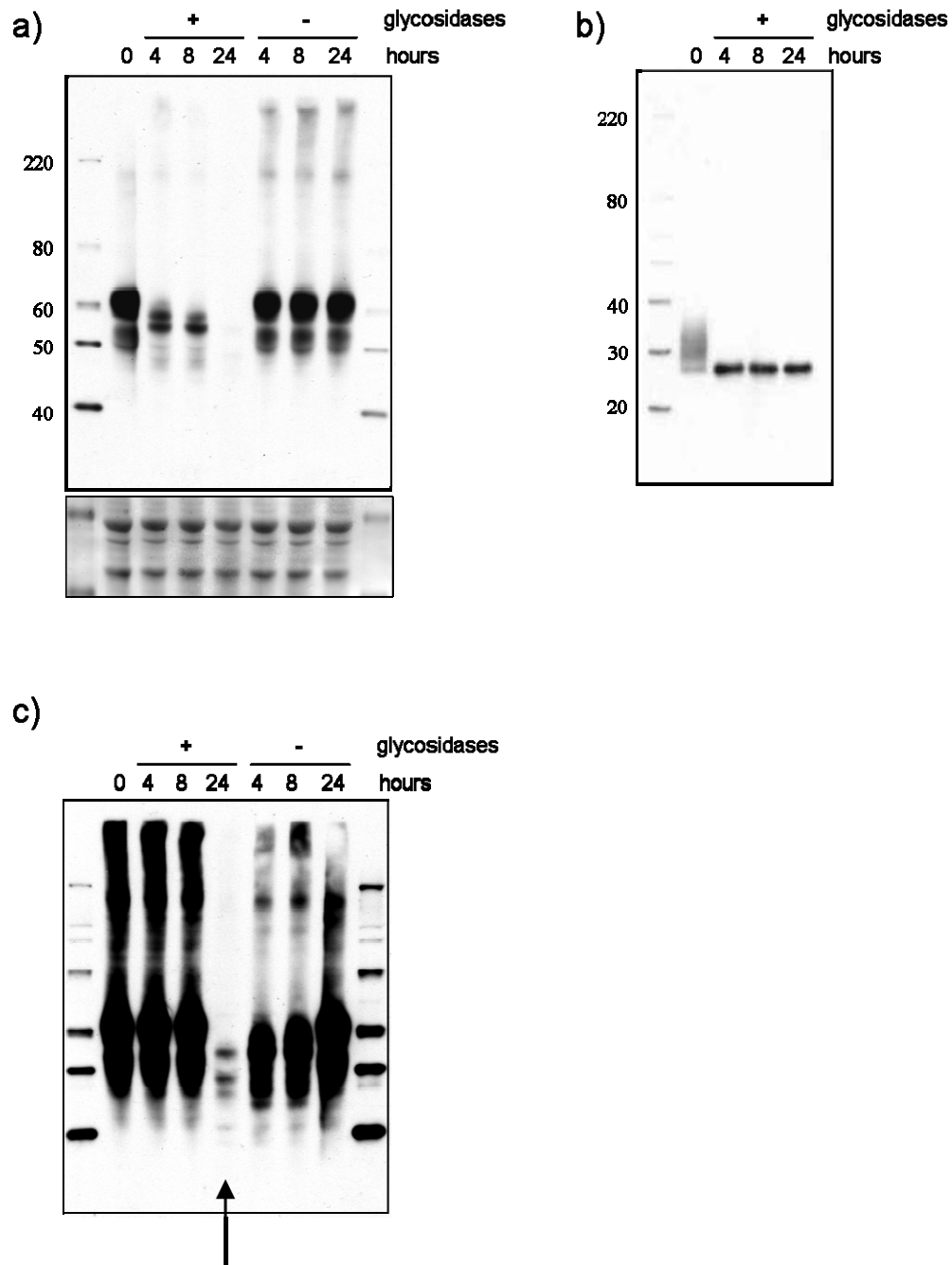


Figure 25. In mouse brain, GlialCAM is glycosylated, the deglycosylated form is rapidly degraded and the protein presumably exists as two splice variants. A) Mouse brain extract that is treated with glycosidases shows band shift to lower Mw and the intensity of the immunoreactive bands decreases with time. B) Purified sGlialCAM at t=0 shows a diffuse band, typical of glycosylated proteins, which condenses to a sharp lower Mw band after treatment with glycosidases. C) When exposing the Western blot for a long time the two major bands at a lower Mw can be detected at t = 24 (see arrow).

2.2.1.2. GlialCAM is phosphorylated in primary rat astrocytes possibly by ERK2 and ASK1

In silico analysis predicts several potential phosphorylation sites in the GlialCAM intracellular domain and Moh et al. showed indirectly that the protein is phosphorylated, however, there was no direct evidence that the protein indeed is phosphorylated (Moh et al., 2005). In order to assess this point, we first determined whether we could immunoprecipitate GlialCAM using our anti-GlialCAM antibody in order to then isolate the protein from cell lysate treated with radioactive phosphate. Lysate from HEK293 cells overexpressing GlialCAM was incubated with the anti-GlialCAM antibody and the protein-antibody complex was precipitated using protein A/G coupled beads. Western blot analysis of the samples showed that GlialCAM can be immunoprecipitated using our antibody, while GlialCAM does not bind to the control antibody (Figure 26a). Furthermore, no GlialCAM immunoreactivity was detected when incubating the GlialCAM or control antibody with lysate from normal HEK293 cells. GlialCAM was not depleted from the lysate with the protocol that was used as the protein could still be detected in the unbound fraction.

Once GlialCAM immunoprecipitation was established we pursued the phosphorylation assay. GlialCAM overexpressing HEK293 cells and primary astrocytes were incubated with [³³P]-orthophosphate, the cells were lysed and GlialCAM was isolated by immunoprecipitation from the cell lysate. The samples were analysed by SDS-PAGE gel electrophoresis, transferred onto a nitrocellulose membrane and [³³P]-orthophosphate incorporation in GlialCAM was determined by autoradiography. As shown in figure 26b, [³³P]-orthophosphate could be detected when immunoprecipitating GlialCAM with the anti-GlialCAM antibody from GlialCAM overexpressing HEK293 cells and primary astrocytes. No radiation was detected when using a control antibody or when incubating lysate of normal HEK cells with the anti-GlialCAM antibody. The bands on the autoradiography correspond to GlialCAM, since a Western blot on the same membrane with anti-GlialCAM antibody detected the same bands (figure 26c). The additional bands detected on the Western blot correspond to the heavy chain of the antibodies used for the immunoprecipitation. Here we showed unambiguously for the first time that GlialCAM is phosphorylated and, interestingly, that it is phosphorylated in cultured primary rat astrocytes. Normalized to the protein amount as seen in the Western blot, the protein in the astrocytes appears to be highly glycosylated.

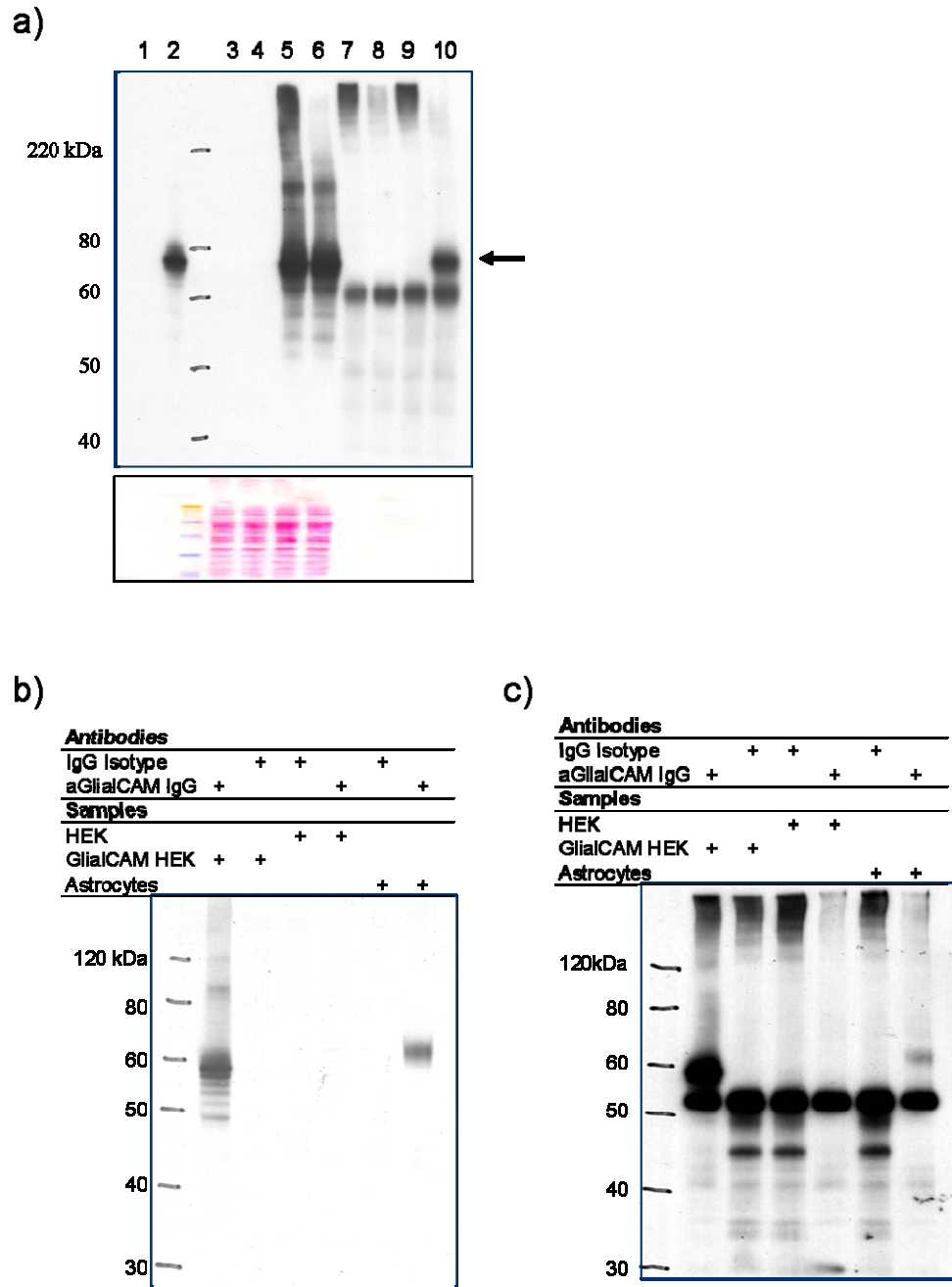


Figure 26. GlialCAM is phosphorylated in astrocytes and in HEK293 cells overexpressing the protein. A) For phosphorylation studies, we first determined that GlialCAM can be immunoprecipitated using the polyclonal anti-GlialCAM antibody. Lysate from HEK cells transfected with the empty plasmid (ctrl lysate) (1) or plasmid containing GlialCAM cDNA (2) was used for the immunoprecipitation (IP). IP with the anti-GlialCAM antibody results in a band corresponding to GlialCAM (arrow) when incubated with GlialCAM containing lysate (10) but not ctrl lysate (9). IP with an IgG isotype control with control (7) or GlialCAM containing (8) lysate did not render GlialCAM bands. Not all GlialCAM was IP:d as GlialCAM can be detected in the unbound fraction after IP with the GlialCAM antibody (6) and the IgG ctrl (5). No GlialCAM is detected in the unbound fraction

from the ctrl lysate (3 and 4). B and C) Normal and GlialCAM overexpressing HEK293 cells, and primary rat astrocytes where incubated with ^{33}P , lysed and GlialCAM was immunoprecipitated from the lysate using the anti-GlialCAM antibody. The samples were analysed by Western blot and exposed on an x-ray film (b) to determine ^{33}P incorporation into GlialCAM and analyzed by Western blot (c) with the anti-GlialCAM antibody. A ^{33}P signal is detected in lysate of HEK293 cells overexpressing GlialCAM (left lane) and primary rat astrocytes (6th lane from the left). Control samples including HEK293 lysate and immunoprecipitation with a rabbit IgG isotype antibody do not show any ^{33}P radiation. The strong band at ca 50 kDa that is detected in all samples in the Western blot corresponds to the antibody heavy chain. The autoradiography was exposed overnight at -70°C , while the Western blot was exposed for 20 seconds (an exposure time at which the radioactive signal is too weak to interfere with the Western blot results).

We wanted to go further and determine which kinases are involved in GlialCAM phosphorylation. For this purpose we cloned and purified the recombinant GlialCAM intracellular domain (GlialCAM-ICD). Originally, we cloned his tagged versions of GlialCAM-ICD with or without the transmembrane domain. However, we were not able to express these constructs in HEK293 cells (data not shown). Therefore, we designed a fusion protein containing the highly expressing $\beta 2$ -microglobulin at the N-terminus followed by a caspase-8 cleavage site, 6-his tag and GlialCAM-ICD. The DNA encoding for the fusion protein was cloned by several consecutive PCR amplifications and transferred into the GatewayTM technology vectors. A signal sequence for protein secretion was added to the 3' end of the open reading frame. The protein was expressed in HEK/EBNA cells and purified from the cell supernatant by metal affinity to Ni^{2+} NTA and size exclusion chromatography (figure 27). The purified fusion protein was cleaved using caspase-8, rendering two separate entities, namely $\beta 2$ -microglobulin and his-tagged GlialCAM-ICD. The latter was purified again through metal affinity chromatography and separated from remaining uncleaved protein through cation exchange chromatography.

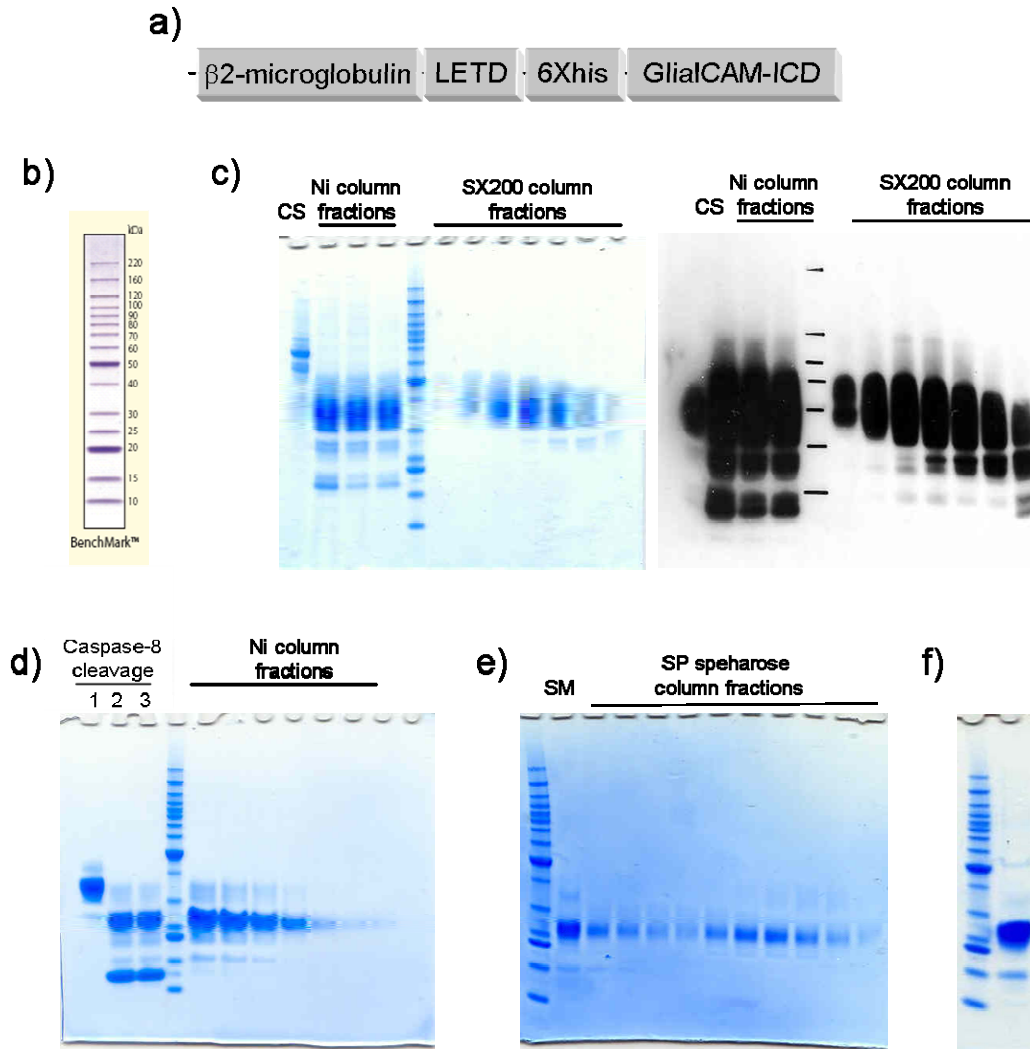


Figure 27. Purification of GlialCAM intracellular domain. a) The cDNA of a recombinant protein containing beta-2-microglobulin (B2MG), followed by a caspase-8 cleavage site (LETD), 6-his tag and GlialCAM intracellular domain (ICD) was cloned into the mammalian expression vector pEAK12d and expressed as an excreted protein in HEK293 cells. b) BenchMark protein standard (invitrogen) that was used for the gels stained with coomassie blue (c, d, e). c) The cell supernatant (CS) was passed through a Nickel column and the bound protein eluted with EDTA. Then, it was run through a size exclusion column (SX200) in order to remove contaminants. The fractions were analysed by SDS-PAGE and coomassie (left image) and by Western blot using an anti-B2MG antibody (right image). d) Fractions containing the protein were pooled together and cleaved with Caspase-8. The left most lane shows the uncleaved protein (lane 1), followed by the protein cleaved for 1 hour (lane 2) and for 2 hours (lane 3). Following cleavage the GlialCAM-ICD-6his residue was isolated using a Nickel column and the EDTA elution fractions containing the protein were pooled. e) To remove contaminants the solution (SM: starting material) was run through a SP sepharose cation exchange column and again the fraction containing the protein of interest (bracket) were pooled together. A G25 coarse size exclusion column was then used to change the protein buffer. f) The final solution was analysed by SDS-PAGE and Simply Blue staining under non-reduced conditions.

The purified his-tagged GlialCAM-ICD was used for *in vitro* kinase assays. The purified protein was incubated with different kinases, ERK2, JNK3, MEK, CK2a, ASK1 and PI3K γ in the presence of radioactive [^{33}P]-ATP. The samples were separated by SDS-PAGE gel electrophoresis and incorporation of radioactive phosphate was analysed by phosphoimaging and autoradiography. As shown in figure 28, a radioactive signal can be detected in the samples in which GlialCAM-ICD was incubated with ERK2 or ASK1, and the position of the band on the gel corresponds to the position previously observed for purified GlialCAM-ICD. MBP was used as a positive control for the assay and the protein is phosphorylated by all the kinases tested, although at varying levels. Most of the kinases auto-phosphorylate rendering a signal that is also present in the negative control containing only the kinase. Whether GlialCAM is also a substrate for ERK2 and ASK1 *in vivo* remains to be determined.

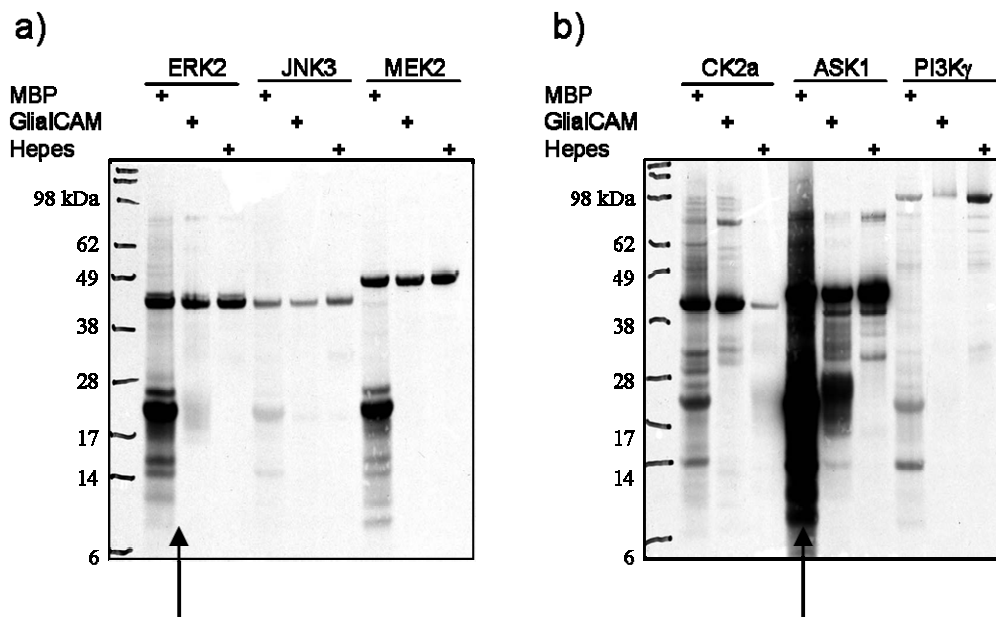


Figure 28. The purified GlialCAM intracellular domain (GlialCAM-ICD) is phosphorylated by ERK2 and ASK1 *in vitro*. GlialCAM-ICD, MBP (ctrl pos) or Hepes buffer alone (ctrl neg) was incubated with different kinases in the presence of γ - ^{33}P -ATP, the samples were separated on an SDS-PAGE gel and radioactivity was detected using a PhosphorImager. GlialCAM-ICD shows ^{33}P incorporation in the presence of ASK1 (arrow, right gel) and ERK2 (arrow, left gel), while MBP is phosphorylated by all the kinases tested and no signal is seen with buffer alone (except for kinase autophosphorylation). ERK2: Extracellular signal-regulated kinase 2; JNK3: c-Jun N-terminal kinase-3; MEK2: MAPK/ERK kinase 2; CK2a: Casein kinase II subunit alpha; ASK1: Apoptosis Signal-regulating Kinase 1; PI3K γ : Phosphoinositide-3 kinase gamma.

2.2.1.3. GlialCAM induces cell clustering while no cis homo dimers were detected

Moh M et al. (2005) conclude that GlialCAM primarily forms cis- compared to trans-homo interactions when overexpressed in the MCF7 breast tumour cell line. We analysed these GlialCAM adhesion properties by overexpressing the protein in HEK293 cells. In contrast to Moh M et al., who used constructs where GlialCAM is tagged with GFP or V5 tag, we used an untagged version of the protein. To study cis-homo interactions, adherent GlialCAM expressing HEK293 cells were dissociated from the culture dish as well as from each other using trypsin and EDTA. Single cell suspension was ensured by visual inspection in a phase contrast microscope. Cells were then incubated with BS3, a non-cleavable, membrane impermeable, water-soluble cross-linker. It contains two amine-reactive N-hydroxysulfosuccinimide (NHS) groups at opposite ends of the molecule, through which it forms stable amide bonds with primary amines. The reaction was stopped by adding Tris and the cells washed before they were lysed and the samples analysed by Western blot. NeCl-1 that has been shown to form cis-homo interactions was used as a control (Kakunaga et al., 2005). As shown in figure 29, we can detect cis oligomerisation of Necl-1, but not GlialCAM. No immunoreactivity for either protein is detected in normal HEK cell.

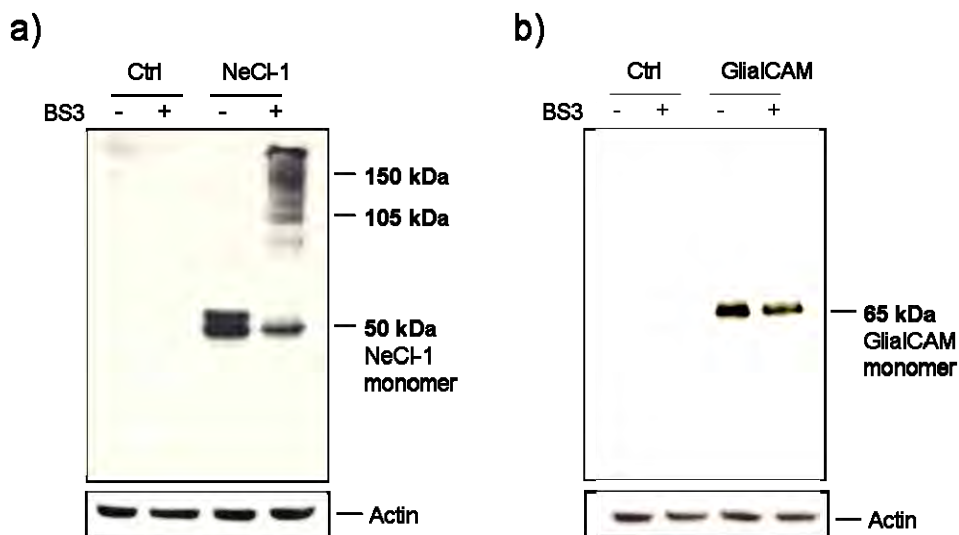


Figure 29. GlialCAM does not form cis dimers when overexpressed in HEK293 cells. HEK cells were transfected with the pEAK12d vector alone (ctrl), containing NeCl-1 (pos ctrl) or GlialCAM. Cell clusters were

dissociated to single cells using trypsin and an aliquote of the cells were treated with Bis(Sulfosuccinimidyl) suberate (BS3) crosslinker. Samples were analysed by Western blot using an anti-NeCl-1 antibody (a) and the anti-GlialCAM antibody (b), respectively. NeCl-1 shows higher order oligomers (high Mw bands on the Western blot) indicating cis oligomerisation, while no higher order oligomers are detected for GlialCAM. HEK cells do not express these proteins.

In order to study trans-homo oligomerisation, we cloned GlialCAM and Necl-1, as well as a negative control, EGFR, into a plasmid that carries a low expression of GFP. Hence, cells expressing the desired proteins could be identified through GFP expression, without having to tag the proteins. NeCl-1 has been previously shown to form trans-homo interactions (Kakunaga et al., 2005), while we did not find any reports on EGFR trans-homo oligomerisation. The newly constructed plasmids successfully express the desired proteins in HEK293 cells as shown in Western blots depicted in figure 30a,b,c. When studying cells transfected with the plasmid alone or containing GlialCAM and NeCl-1 cDNA by fluorescence and phase contrast microscopy we observed that GlialCAM and NeCl-1 expressing green cells formed clusters while surrounding non-labelled cells maintained a flat single cell layer appearance (figure 30d). Cells transfected with the GFP plasmid without insertion remained as a flat single cell layer without any noticeable clustering.

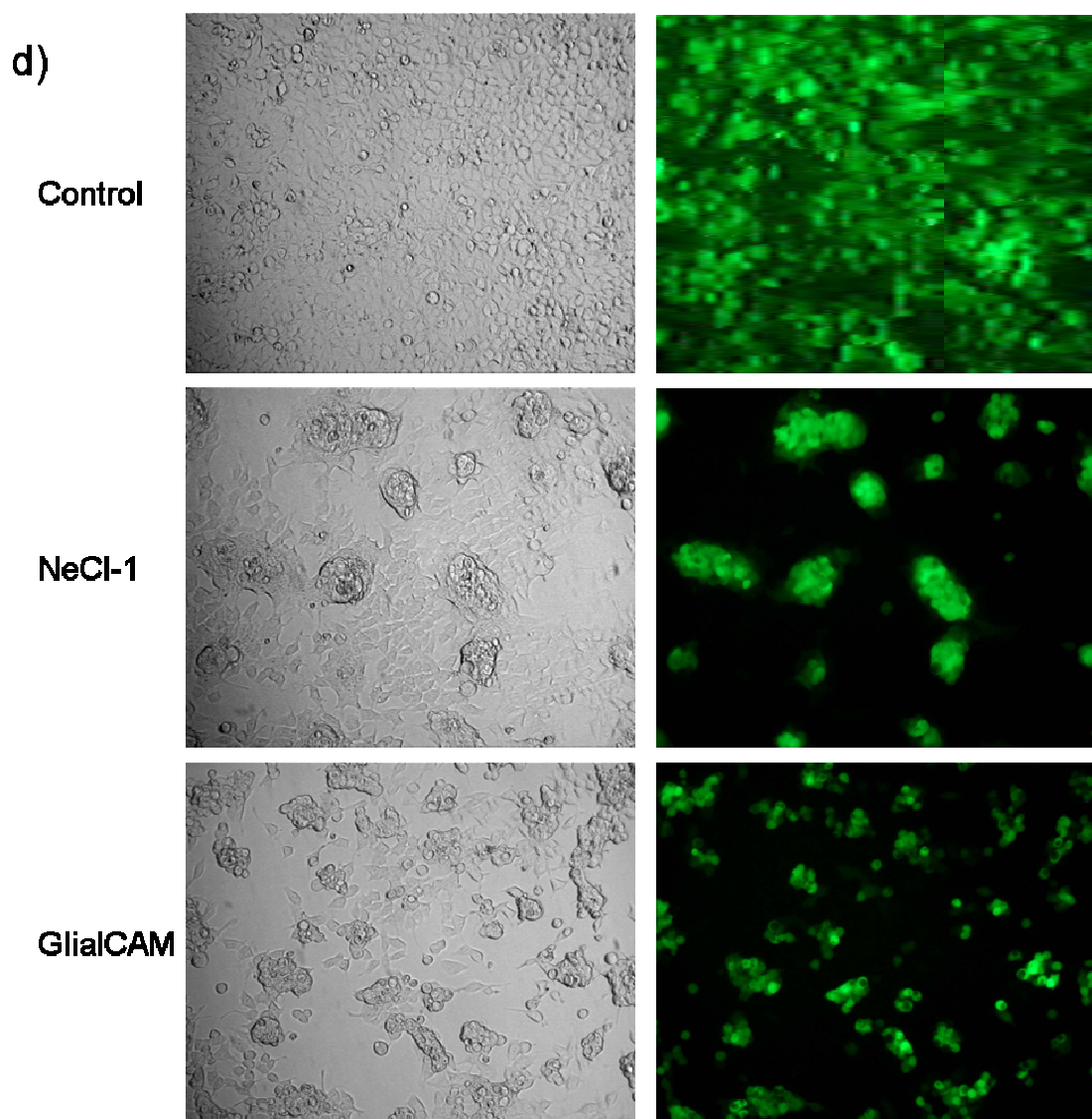
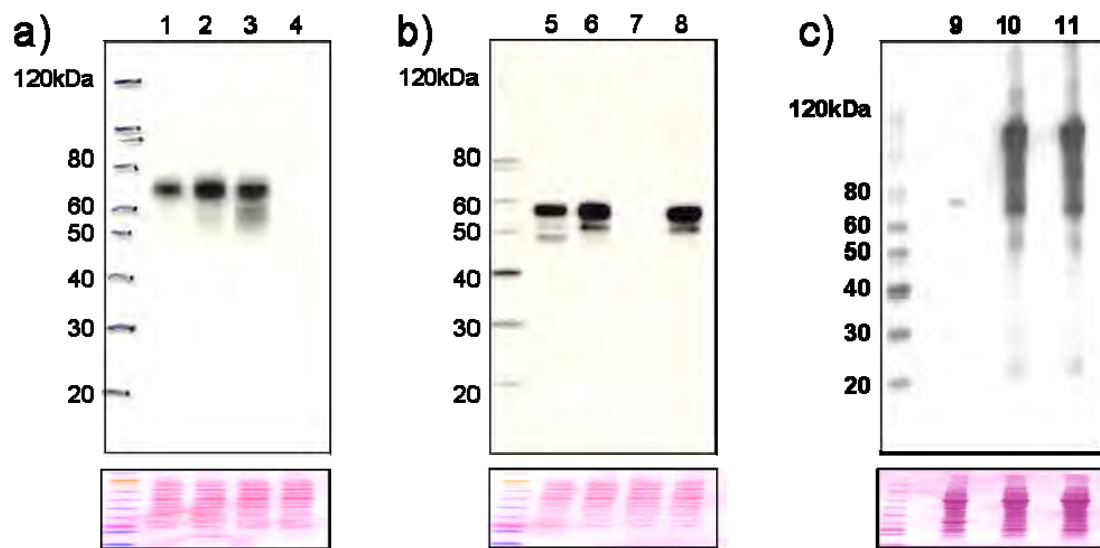


Figure 30. GlialCAM overexpression induces clustering of HEK 293 cells. A) For the experiment, GlialCAM, NeCl-1 and EGFR cDNAs were cloned into pEAK12d plasmids, respectively, which express eGFP with a separate and weaker promoter than the inserted cDNA. The plasmids were transfected into HEK293 cells and protein expression was determined by SDS-PAGE and Western blot. A) An Western blot with the anti-GlialCAM antibody reveals that GlialCAM is expressed with this plasmid construct after 24 h (1), 48 h (2) and 72 h (3) transfection, no immunoreactivity was detected in cells transfected with the plasmid expressing NeCl-1 (72 h transfection) (4). B) An Western blot with the anti-NeCl-1 antibody reveals that NeCl-1 is expressed with this plasmid construct after 24 h (5), 48 h (6) and 72 h (8) transfection, no immunoreactivity was detected in cells transfected with the plasmid expressing GlialCAM (72 h transfection) (7). C) An Western blot with the anti-EGFR antibody reveals that 48 h after transfection EGFR is expressed with this plasmid construct (11) and EGFR cDNA in pEAK12d without GFP (10), and trace expression was detected HEK cells transfected with the empty plasmid (9). D) Adherent HEK 293 cells were transfected with the pEAK12d plasmid expressing eGFP alone or together with GlialCAM or Necl-1 and grown for 48 h. No clustering was observed when transfecting the cells with eGFP alone (negative control), while clustering was observed when transfecting the cells with eGFP together with NeCl-1 (positive control) or GlialCAM.

Then, we transfected suspension HEK/EBNA cells for a clustering assay. The cells were transfected for 48 h with plasmid alone or containing GlialCAM, NeCl-1 or EGFR, before they were treated with a non-enzymatic cell dissociation solution to obtain a single cell suspension. We decided to use the non-enzymatic solution to avoid that trypsin would cleave the extracellular domain of GlialCAM and hence interfere with the results, even though in most published cell aggregation studies trypsin is used for cell dissociation. Single cell suspensions were incubated at 37°C under gentle shaking for 20 min and amount of cell clustering was compared to cells directly after dissociation by fluorescence and phase contrast microscopy (figure 31). HEK/EBNA suspension cells show significant clustering when transfected with plasmids containing GlialCAM or NeCl-1. Thus, it was hard to disrupt these clusters with the cell dissociation medium without harming the cells and therefore even at the beginning of the incubation small clusters were present in the GlialCAM or NeCl-1 expressing cell samples, whereas EGFR and control transfected cells were closer to single cell suspension. However, considerably more cell clustering was seen after 20 min incubation in cells expressing GlialCAM or NeCl-1 compared to cells expressing EGFR or transfected with an empty vector. We were unable to disrupt GlialCAM induced cell clusters through addition of sGlialCAM (data not shown), we believe it is because it is hard to disrupt the abundant clustering caused by GlialCAM overexpression, which might involve multiple strong homotypic interactions in tight junctions between the cells.

To conclude, GlialCAM expression enhances HEK cell aggregation. Homotypic interactions is supported by the observation that mainly transfected cells clustered, whereas untransfected cells continued to grow as flat cell layers and remained as single suspension cell. Furthermore, in our hands and with our protocol GlialCAM does not form cis-homo interactions.

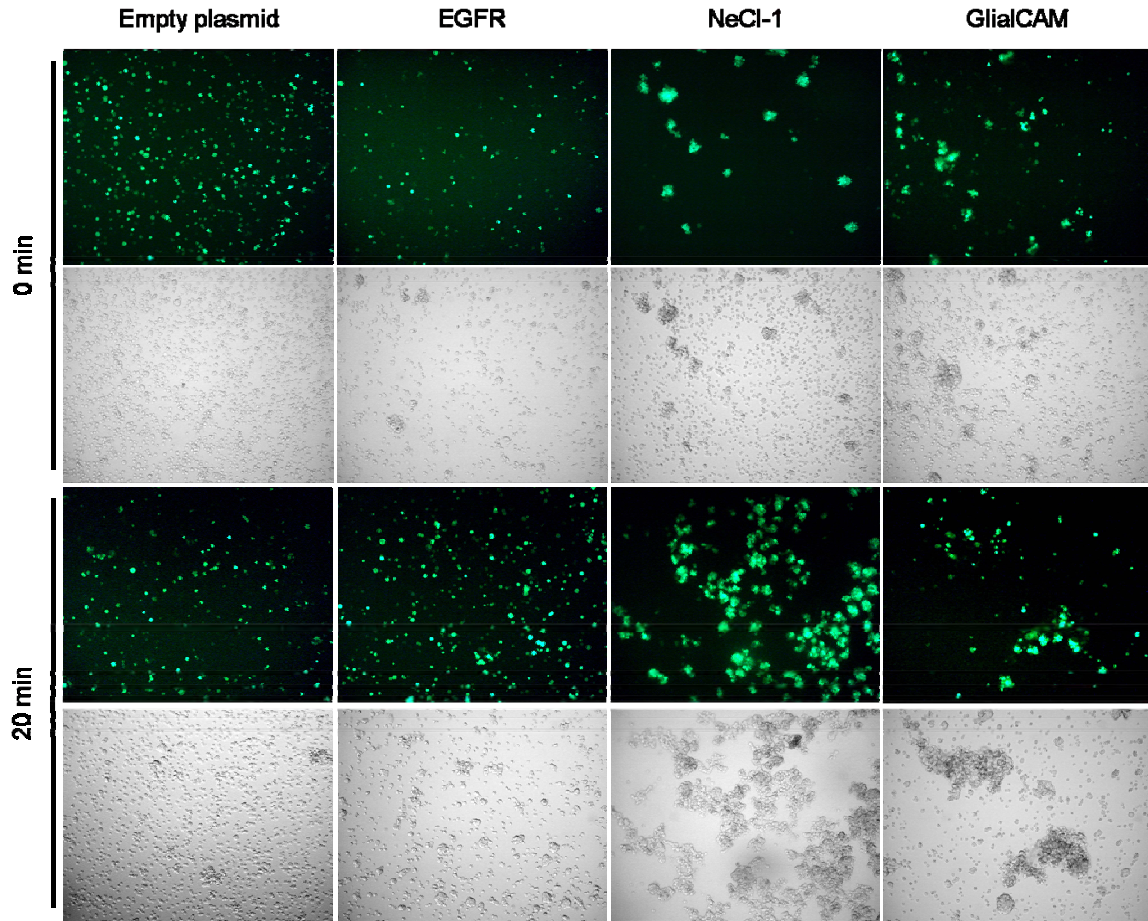


Figure 31. GlialCAM overexpression induces clustering of HEK 293 cells in suspension. HEK 293 cells in suspension were transfected for 72 h with the pEAK12d plasmid expressing eGFP alone or together with GlialCAM or Necl-1 or EGFR. Clusters in the cell suspensions were dissociated using the cell dissociation solution (C5789, Sigma) after which the cells were incubated for 20 min on a rocking platform in Hank's buffered salt solution. The upper panel shows the cells directly after the dissociation and the lower panel shows the cells after the 20 min incubation. No or little clustering was observed of the cells transfected with eGFP alone (negative control) or together with EGFR (negative control), while clustering was observed of the cells transfected with eGFP together with NeCl-1 (positive control) or GlialCAM.

2.2.1.4.sGlialCAM together with PDGF-AA-A induces OPC migration in an agarose drop migration assay

We have demonstrated (see section on GlialCAM expression) that GlialCAM is expressed in all maturation stages of OLs *in vitro*. In OPCs, we specifically detected the protein in the cell body and in growth cone-like structures where it co-localises with GAP43. OL growth cone-like structures are highly dynamic entities that are important for directional cell motility and cell-cell and cell-ECM contacts (Fox et al., 2006). This finding suggests a possible role of GlialCAM in cell motility and therefore we decided to study whether GlialCAM is involved in OPC migration. We chose a well described OPC migration assay, where the cells are placed in an agarose drop at a high density in the middle of a cell culture dish and migration out of this drop is monitored by phase contrast microscopy. The advantages of this assay compared to a transwell migration assay is that cell motility can be continuously monitored, while in with the transwell migration assay only one timepoint can be measured for each set of cells (Frost et al., 2000). Furthermore, the morphology of the migrating cells can be examined and activating or inhibitory agents can be added or removed during the course of the assay. However, while with a transwell migration assay the experiment is fairly rapid and migration can be detected within 8 h, the agarose drop migration assay generally takes 24-72 h. The latter assay requires less cells, which is an advantage when working with primary cells, but on the other hand it requires higher quantities of test molecules. One has to keep in mind that, during the course of the assay, cell proliferation may affect migration and also OPCs will synthesise and secrete their own ECM in addition to the ECM studied. We set the assay up using Oli-*neu* cells and carefully following the protocol described by Milner R et al. (Milner et al., 1996). Oli-*neu* is a stable cell line generated from primary mouse OLs by inserting the t-*neu* oncogene, which results in constitutive activation of tyrosine kinase and hence oncogenic properties (Jung et al., 1995). First we studied Oli-*neu* migration on fibronectin coating with and without PDGF-AA and observed that unlike OPCs (Milner et al., 1996), Oli-*neu* cells do not show increased migration from the drop in the presence of PDGF-AA (figure 32a), which can be related to the finding that PDGF alone does not induce Oli-*neu* proliferation (Strelau and Unsicker, 1999). We then studied Oli-*neu* migration on different coated surfaces and we observed migration on fibronectin, laminin, poly-L-ornithine, poly-D-lysine and matrigel, whereas no migration was detected on uncoated cell dishes (plastic) (figure 32b). The influence of the size of the agarose drop on migration was also examined, especially to explore the possibility to

use smaller drops and hence less cells per condition. As shown in figure 32c, with a constant cell density, the size of the drop influences migration. The 1.5 μl drop size that is used by Milner et al. seems to be optimal, since with a 2.5 μl drop size we observe less migration and with a 0.5 μl drop size we detected no or very little migration. This finding was a bit surprising and the only explanation we could find is that the observed phenomenon could be due to the surface tension of the drop, where a high surface tension (especially in the 0.5 μl drop) could prevent the cells from migrating out. However, this remains pure speculation.

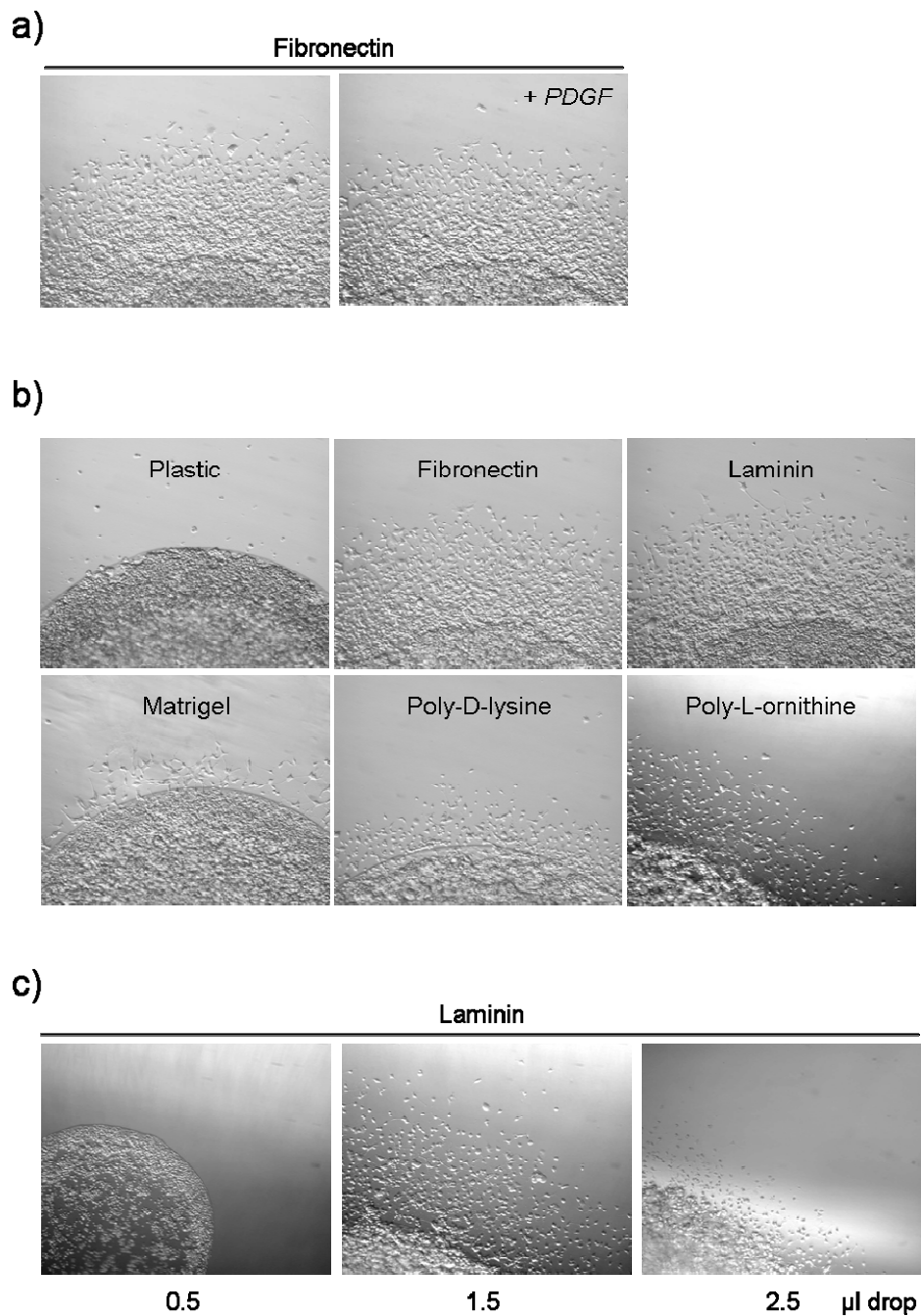


Figure 32. Agarose drop migration assay was set-up using Oli-*neu*. Migration from the drop was studied by phase contrast microscopy. a) No detectable difference of Oli-*neu* cell migration out of the drop was seen on fibronectin coating with (lower image) or without (upper image) PDGF-AA. b) Oli-*neu* migration can be detected on fibronectin, laminin, poly-L-ornithine, matrigel and poly-D-lysine, while no migration is seen on plastic. c) With a constant cell density of 40×10^6 cells/ml, a 1.5 µl drop renders more migration than a bigger, 2.5 µl, or a smaller, 0.5 µl, drop. Migration was studied on ornithine coating. These are representative images from two independent experiments.

Once the method was established with the *Oli-neu* cell line, we applied it to primary rat OPCs. Of the various coatings tested, these cells migrated best on poly-L-ornithine and poly-D-lysine coating (figure 33a). Some migration was also detected with a matrigel coating, while no migration was seen from drops placed on uncoated culture dish (plastic) or collagen. Migration was dependent on the presence of PDGF-AA, except for matrigel coating where some migration could also be seen in the absence of PDGF-AA. These findings are in agreement with previous reports (Milner et al., 1996). Furthermore, we found that there is a threshold of cell density in the agarose drop for PDGF induced migration: with 40×10^6 cells /ml in the drop we observed migration out from the drop while with half of that cell density no or little migration was observed (figure 33b). Some possible explanations to this phenomenon might be that the cells require a certain density to remain viable or migration might be triggered only above a certain cell density (Frost et al., 2000).

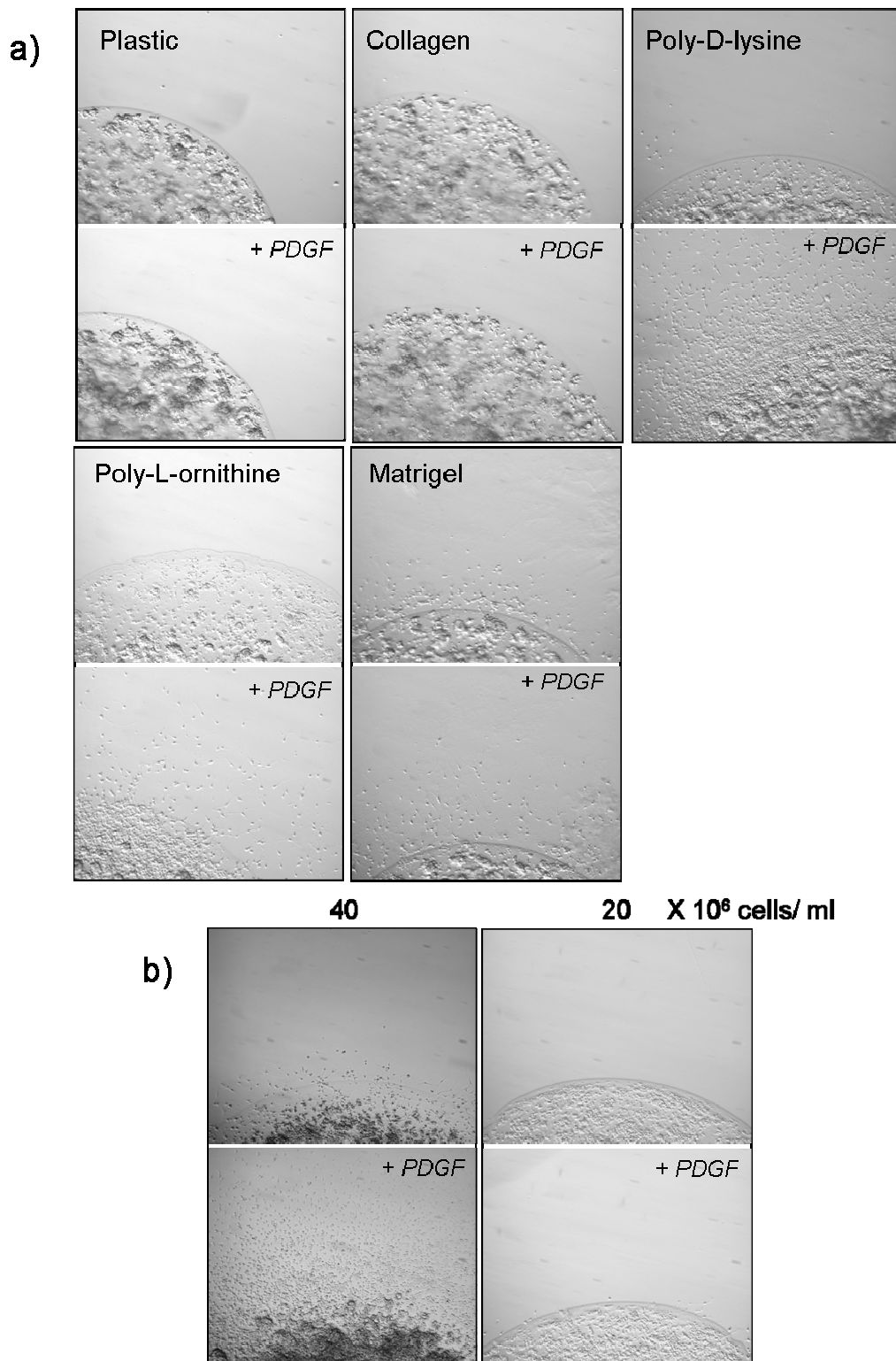


Figure 33. OPCs migration in the agarose drop assay is dependent on the cell dish coating and the concentration of cells in the drop. a) After 72 h in culture, no or very little migration is observed in the absence of PDGF-AA. In the presence of PDGF-AA migration is observed on poly-D-lysine, poly-L-ornithine and matrigel coating, while no migration is detected on uncoated (plastic) or collagen coated dishes. B) After

96 h in culture, no or little migration is detected, with or without PDGF-AA, from drops containing 20×10^6 cells/ ml. On the contrary, with 40×10^6 cells/ ml in the drop, PDGF-AA induces OPC migration from the drop and some migration can also be detected without PDGF-AA. The images are representative from two independent experiments.

To test the possible role of GlialCAM in OPC migration, we examined the effect of sGlialCAM. As demonstrated earlier, GlialCAM forms homo-trans oligomers. Moreover, sGlialCAM in solution is predominantly found as a dimer (data not shown). Thus, there are reasons to believe that sGlialCAM could interfere with GlialCAM mediated interactions between cells and affect migration. To study the effect of sGlialCAM, agarose drops containing 40×10^6 cells /ml were prepared, placed on poly-L-ornithine or poly-D-lysine coating and cultured in the presence or absence of sGlialCAM. As shown in figure 34, while PDGF-AA induced migration, we did not see a clear difference in migration between sGlialCAM treated and non-treated cells, either in the presence or absence of PDGF-AA on any of the two coatings that were tested.

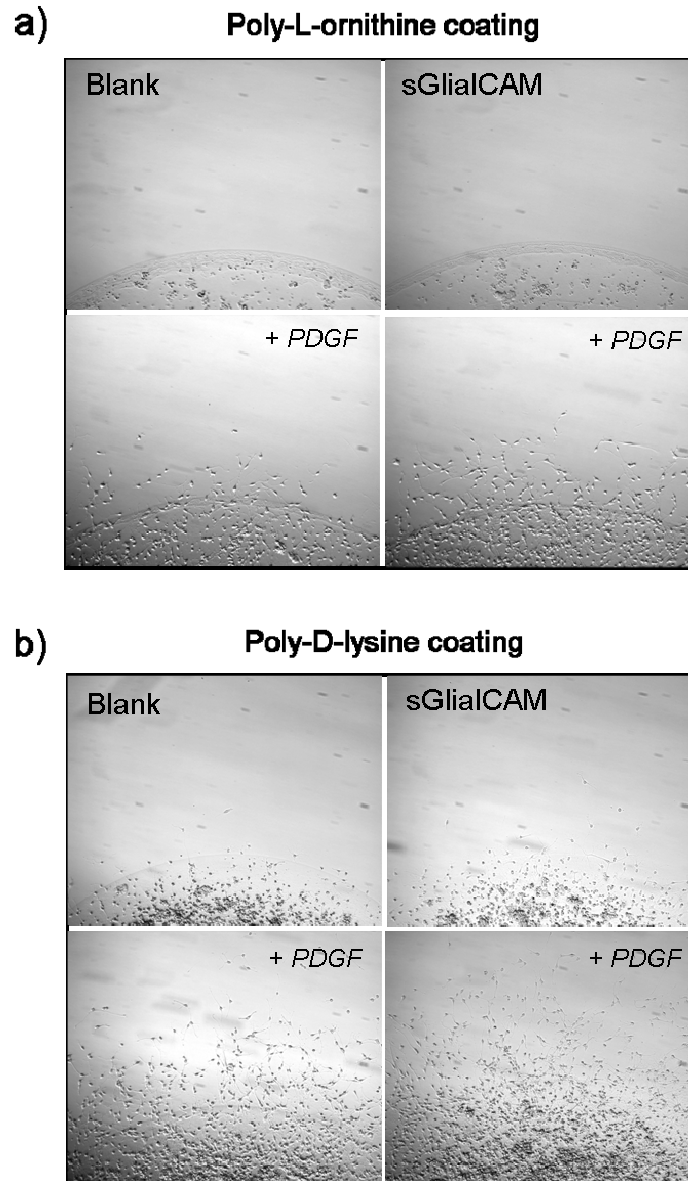


Figure 34. With 40×10^6 cells/ ml in the agarose drop, there is no evident difference in OPC migration in the presence or absence of sGlialCAM, either on poly-L-ornithine (a) or poly-D-lysine. PDGF-AA induces OPC migration on both surfaces.

However, when we reduced the cell density in the agarose drop to 20×10^6 cells /ml, which was below the established threshold cell density to observe migration with PDGF, there was a striking difference in migration between sGlialCAM treated and non-treated cells in the presence of PDGF-AA (figure 35). For these experiments, we used his-tagged sCD147 as a negative control, because this protein is about the same size and contains two Ig-like domains like sGlialCAM, without being so closely related that similar

activities could be expected. Neither were we able to find any reports on sCD147 affecting OPC migration.

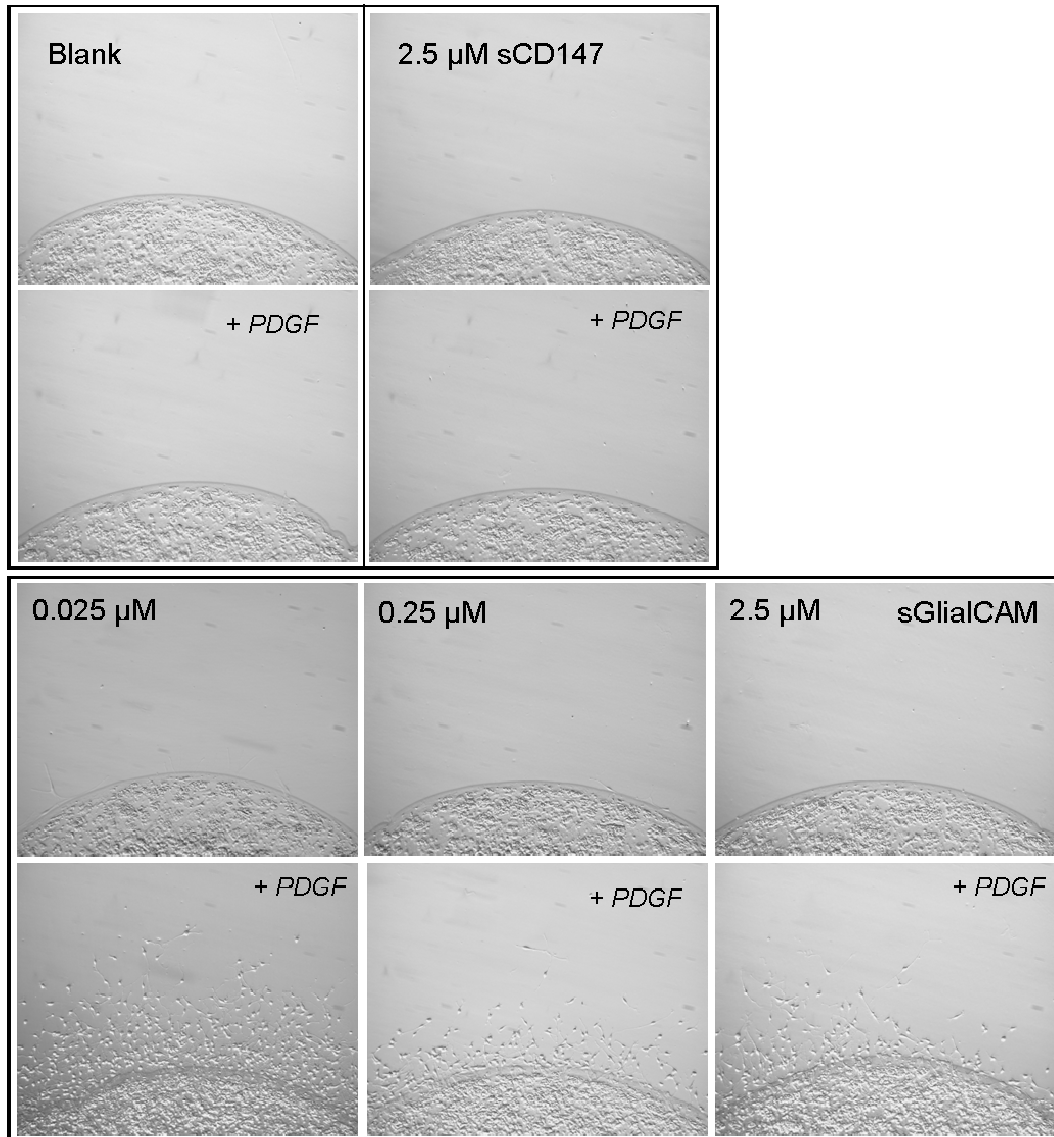


Figure 35. In the presence of PDGF-AA sGliaCAM enhances OPC migration from the agarose drop assay. A) Light microscope image of OPC migration from drops treated with both sGliaCAM and PDGF-AA at the 3 tested concentrations (lower panel), while little or no expression is seen PDGF-AA alone (upper left panel) or together with the control protein sCD147 (upper right panel).

We observed that OPCs did not migrate as a uniform corona out from the agarose drop and hence to quantify the migration we took several images, covering the whole

periphery of the drop, with a camera connected to the microscope. The images were handled and analysed using an image processing software that was adapted by the bioinformatics group in the institute from the ImageJ program of NIH. The different steps of the analysis are presented in figure 36. First, images were merged in order to reconstitute the drop, then the background in the images were normalised and finally OPCs were detected through the bright contrast of their round cell bodies. A threshold for the size of the bright spot was determined, in order to specify unique cells in clusters and to disregard any contaminating debris in the culture. Both median distance of the OPCs from the periphery of the agarose drop, as well as the total amount of cells that had migrated out from the drop were determined.

As shown in the graphs of figure 36, sGlialCAM together with PDGF-AA enhanced both the median distance covered by the OPCs from the edge of the drop (left graph) as well as the number of OPCs outside the drop (right graph). sGlialCAM without PDGF-AA did not significantly enhance migration. We tested three different concentrations of sGlialCAM, 0.025, 0.25 and 2.5 μ M, in order to study the dose response. However, after a primary strong increase in OPC motility with 0.025 μ M sGlialCAM, we obtained a negative correlation between sGlialCAM concentration and migration with the higher concentrations (both for median distance and cell count). The dose response resembles the response observed by Neuregulin-1 β ectodomain enhanced glioma cell migration (Ritch, P, jbc, 2003). Nevertheless, with the highest concentration of sGlialCAM tested together with PDGF-AA, the cells still migrate significantly further than with PDGF-AA alone or PDGF-AA and sCD147. When looking at the amount of cells moving out from the drop, only 0.025 μ M sGlialCAM together with PDGF-AA showed significant statistical difference. Drops treated with sGlialCAM but not PDGF-AA had a tendency to show OPCs moving further from the drop than untreated and sCD147 treated drops, even though this was not statistically relevant. Taken together, sGlialCAM in conjunction with PDGF-AA clearly enhances OPC migration in the agarose drop migration assay.

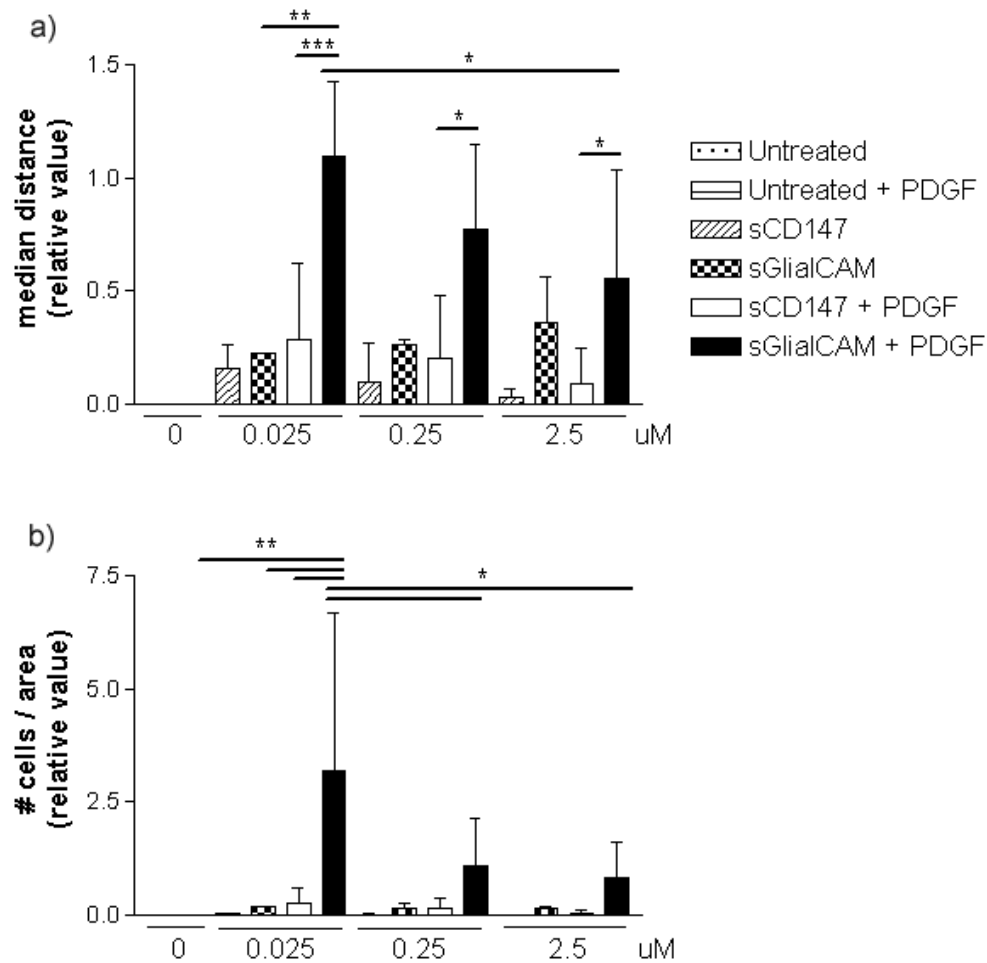


Figure 36. sGliaICAM enhances OPC migration in the agarose drop migration assay. a) The median distance travelled by OPCs from the agarose drop is greater in the presence of sGliaICAM and PDGF-AA compared to PDGF-AA alone (striped bars, no migration) or together with the control protein sCD147 (white bars). This is seen with three different concentration of sGliaICAM. At the concentration range tested there seems to be an inhibitory effect with increase concentrations. b) A similar effect is seen when counting the total amount of cells outside the agarose drop. However, here the higher concentrations of sGliaICAM do not show a significant increase compared to controls. PDGF-AA was added at 5 ng/ml. The results represent three independent experiments and are presented with standard deviation. The data was analysed using Bonferroni's Multiple comparison test, where *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$

Milner et al. (Milner et al., 1997) demonstrated that the distance travelled by the OPCs from the agarose drop is independent of proliferation, however, to exclude the possibility that the observed effect of sGliaICAM is due to enhanced proliferation, we assessed the proliferation rate of each condition by monitoring [3H]-thymidine

incorporation. The agarose drop assay was performed as above and [^3H]-thymidine was added for the last 24 h. Incorporation of radiolabelled-thymidine into the cellular DNA was measured by harvesting the samples onto a glass fiber filter and analysing them with a scintillation counter as described in the methods section. As shown in figure 37, sGlialCAM did not increase proliferation compared to the untreated or sCD147 treated OPCs in the agarose drop, with or without the presence of PDGF-AA. Proliferation was studied on OPCs after 24 h and 48 h in the drop, to exclude a potential initial boost of proliferation that then levels out. PDGF enhanced OPC proliferation in this assay, as previously reported (Milner et al., 1997), and the amount of proliferation increased from 24 h to 48 h. No increase in proliferation was detected between 24 h and 48 h in the drop in the absence of PDGF-AA for all the samples tested.

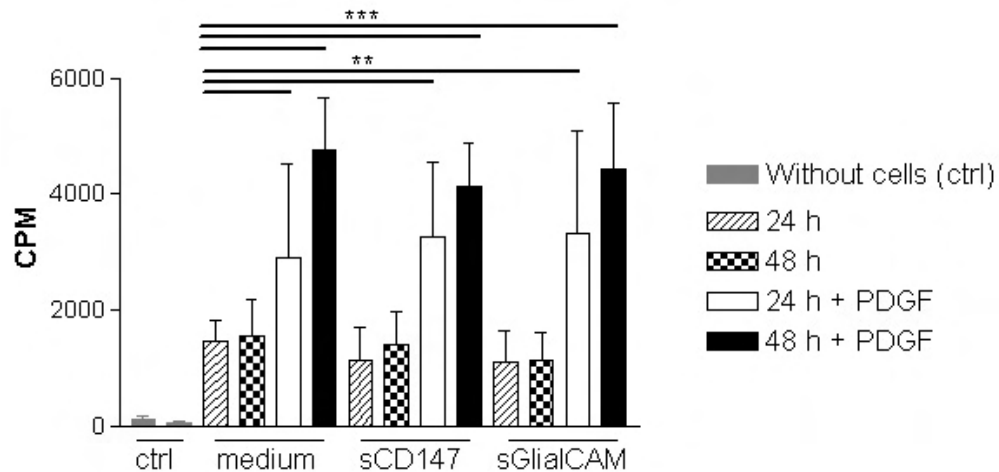


Figure 37. sGlialCAM does not enhance PDGF-AA induced proliferation in the agarose drop migration assay. PDGF-AA induces proliferation in all conditions with a significant enhancement already at 24 h and a further increase at 48 h. There is no additional increase in proliferation when sCD147 or sGlialCAM is added, under the conditions tested. Proliferation was studied using the incorporation of ^3H -thymidine into the cellular DNA and measured as counts per minute (CPM) using a liquid scintillation analyser. The data represents three independent experiments, six drops each, and are presented with standard deviation. The data was analysed using Bonferroni's Multiple comparison test, where *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$

In order to further study the role of GlialCAM in OPC migration, we isolated OPCs from GlialCAM deficient mice (see Favre-Kontula, L., *Glia* 2008) and compared their migratory properties to OPCs isolated from littermate mice expressing GlialCAM at

normal levels, in the agarose drop migration assay. In house results have shown that purified mice OPCs do not respond to PDGF-AA in the agarose drop migration assay (data not shown), presumably because the cells have been amplified in the presence of PDGF-AA and bFGF during the isolation process and with time lose their responsiveness to PDGF-AA (Bogler et al., 1990). However, bFGF induces migration of mouse OPCs in the agarose drop assay and was used in this experiment. Examination of the cells by phase contrast microscopy, showed no striking difference in the morphology of GlialCAM deficient and WT OPCs in culture (Figure 38a), but this would require more extensive examination. In the agarose drop assay experiment we compared the migratory properties of GlialCAM deficient versus WT OPCs. With the higher cell concentration (40×10^6 cells /ml) in the agarose drop, GlialCAM deficient OPCs migrated at similar levels as WT littermate controls, in the presence or absence of bFGF, from the drop (figure 38b). At the same cell concentration there was no evident additional migratory effect of sGlialCAM on rat OPCs, which seems to be consistent with the results obtained with the GlialCAM deficient OPCs. With the lower concentration of cells (20×10^6 cells /ml) in the agarose drop, in the presence of bFGF, we observed more migration with GlialCAM deficient OPCs compared to WT littermates (figure 39c). At the same cell concentration, there was a clear pro-migratory effect of sGlialCAM on rat OPCs, which suggests that sGlialCAM does not have a pro-migratory effect that is dependent on GlialCAM. These preliminary results were obtained from a single experiment with cells isolated from only two WT and GlialCAM $-/-$ mice, respectively, but they seem to indicate a direct function of GlialCAM in OPC migration and clearly deserve further investigation.

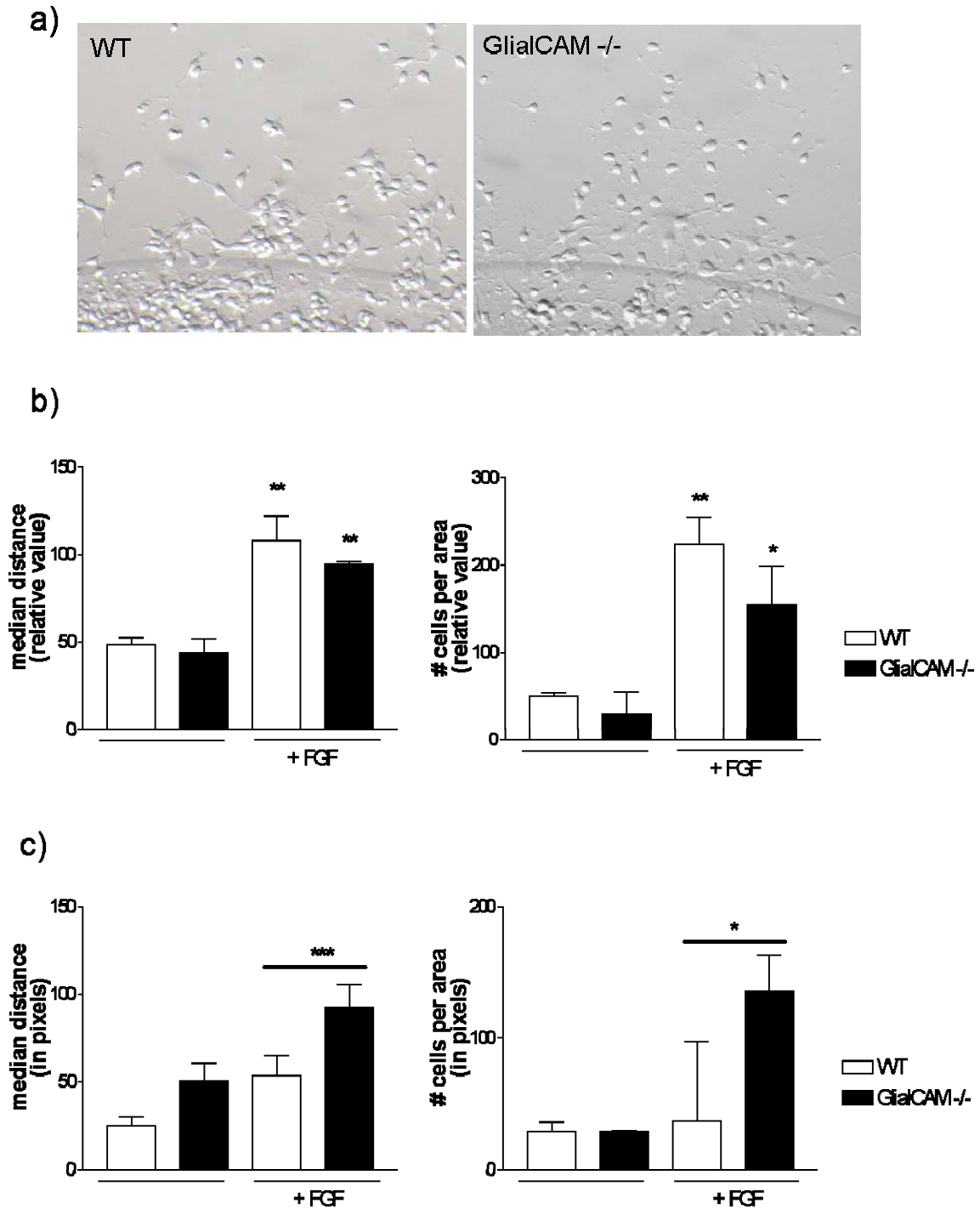


Figure 38. GlialCAM is not essential for OPC migration in the agarose drop assay, however there is an indication that GlialCAM ^{-/-} migrate more than wildtype OPCs. A) Light microscope image of WT and GlialCAM deficient OPCs migrating from an agarose drop. B) At a cell density of 40×10^6 cells/ml in the drop, WT and GlialCAM deficient OPCs, both in the presence and absence of bFGF, migrate the same median distance (left graph) from the drop and a similar number of cells are detected (right graph) outside

the agarose drop. bFGF enhances migration in both cell populations in this assay and the asterix show statistical difference to the same cell type without bFGF. C) When using half the cell density in the drop (20×10^6 cells/ml), in the presence of bFGF GlialCAM $-/-$ OPCs migrate further and at larger numbers than WT OPCs. There is a tendency that GlialCAM $-/-$ OPCs migrate further than their WT counterparts even without bFGF, even though this is not statistically significant. The data represents triplicates in a single experiment and are presented with standard deviation. The data was analysed using Bonferroni's Multiple comparison test, where $**p < 0.01$, $*p < 0.05$.

2.2.1.5. GlialCAM is downregulated in astrogliomas and oligodendrogliomas

As mentioned above, it has been demonstrated that GlialCAM is downregulated in human hepatocellular carcinoma (Chung et al., 2005) and the gene is mapped to the human chromosome 11q24, a loci which is frequently correlated with carcinogenesis (Koreth et al., 1999). Furthermore, CAMs are frequently deregulated in tumours. For these reasons, we decided to study whether GlialCAM expression is altered in astrocyte and OL tumours. Snap frozen brain tumour tissues were received from the Canadian Brain Tumour Tissue Bank. All the tumour tissues had been clinically characterised and graded and the supplier had determined the quality of the sample by H&E (hematoxylin & Eosin) staining. All the tissue samples were graded good quality (grading system includes good, moderate and poor), meaning a high ratio of tumour cells compared to hemorrhagic, necrotic and fibrous tissue contaminants, and they were all from the tumour center. The tissues were homogenised and the lysates were analysed by gel electrophoresis and Western blot. Since GlialCAM is mainly expressed in OLs and astrocytes, we used human cerebellum that expresses GlialCAM at high levels as control for GlialCAM expression in oligodendroglioma and astrocytoma (Favre-Kontula et al. *Glia*, 2008). Figure 39a shows the quantification of GlialCAM normalized to actin on Western blots. For each group, 12 individual tumours were analyzed and the results presented in the figure show the average with standard deviations. GlialCAM expression is significantly downregulated in oligodendroglioma and astrocytoma compared to the control. GlialCAM is expressed at lower levels in the higher (grade III) compared to the lower tumorigenicity grade oligodendroglioma (grade II), while astrocytoma grade I and glioblastoma show similar expression levels. Furthermore, decreased levels of GlialCAM expression is detected in human glioblastoma cell lines, A-172 and U-251, compared to human and rat primary astrocytes (figure 39b).

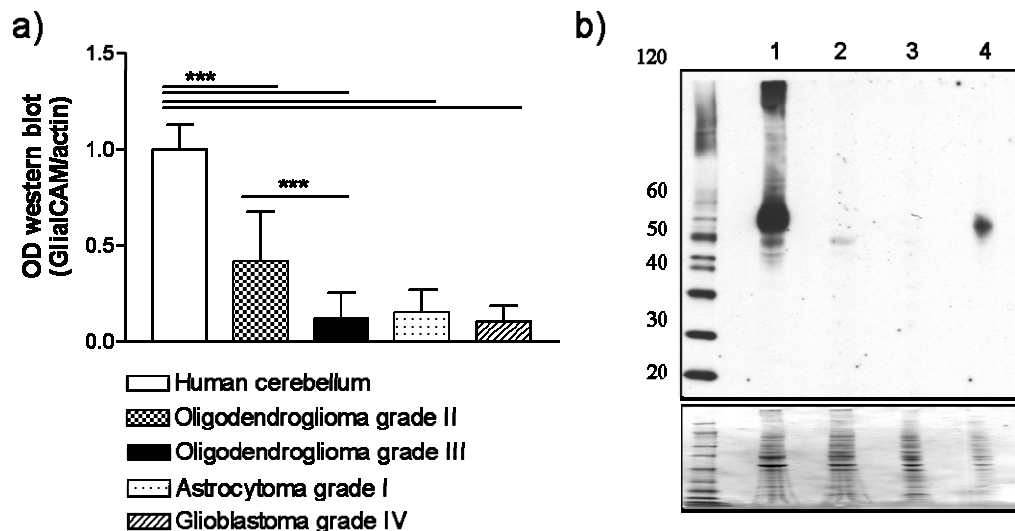


Figure 39. GlialCAM is downregulated in oligodendroglioma and astrocytoma. A) Western blot analyses of GlialCAM expression in human brain tumours shows that GlialCAM expression levels inversely correlate with oligodendroglioma and astrocytoma tumorigenicity. Human cerebellum is used as control. B) Western blot analysis of GlialCAM expression in human glioblastoma (astrocytoma grade IV) cell lines, A-172 (lane 2) and U-251 (lane 3) show that the protein is expressed at lower levels in these cells compared to primary human (lane 4) and rat (lane 1) astrocytes.

Hypermethylation is frequently involved in silencing of various tumour-related genes (Kanai and Hirohashi, 2007). In order to determine whether the low levels of GlialCAM expression in glioblastoma cell lines is due to hypermethylation of the GlialCAM gene, we treated A-172 cells with 5-azacytidine (AzaC), a nucleoside analog that inhibits DNA methyltransferases and thereby DNA methylation. The cells were incubated with 1 μ M or 10 μ M AzaC for 24 h and the cells were subsequently grown for an additional 24 or 48 h in the absence of AzaC, thus for a total of 48 h or 72 h in culture, respectively. The cells were then lysed and analysed by Western blot. Enhanced GlialCAM expression was detected at the end of the 24 h incubation, for both 1 μ M and 10 μ M AzaC treatment, compared to untreated cells (figure 40a). Twenty-four hours after the AzaC treatment GlialCAM expression levels returned to levels comparable with the control. High concentrations of AzaC can be toxic to the cells and indeed we observed cellular toxicity after 24 h with 10 μ M AzaC, while after 24 h of 1 μ M AzaC treatment, cells appeared similar to the controls (figure 40b). We repeated the experiment with 3 μ M concentrations of AzaC. At this concentration no toxicity was seen (data not shown). At

the end of the 24 h treatment a slight increase in GlialCAM expression was observed and after further 24 h incubation in the absence of AzaC, the increase was even more pronounced and statistically significant compared to untreated samples (figure 40c). These results suggest that GlialCAM expression is downregulated in glioblastoma through DNA hypermethylation.

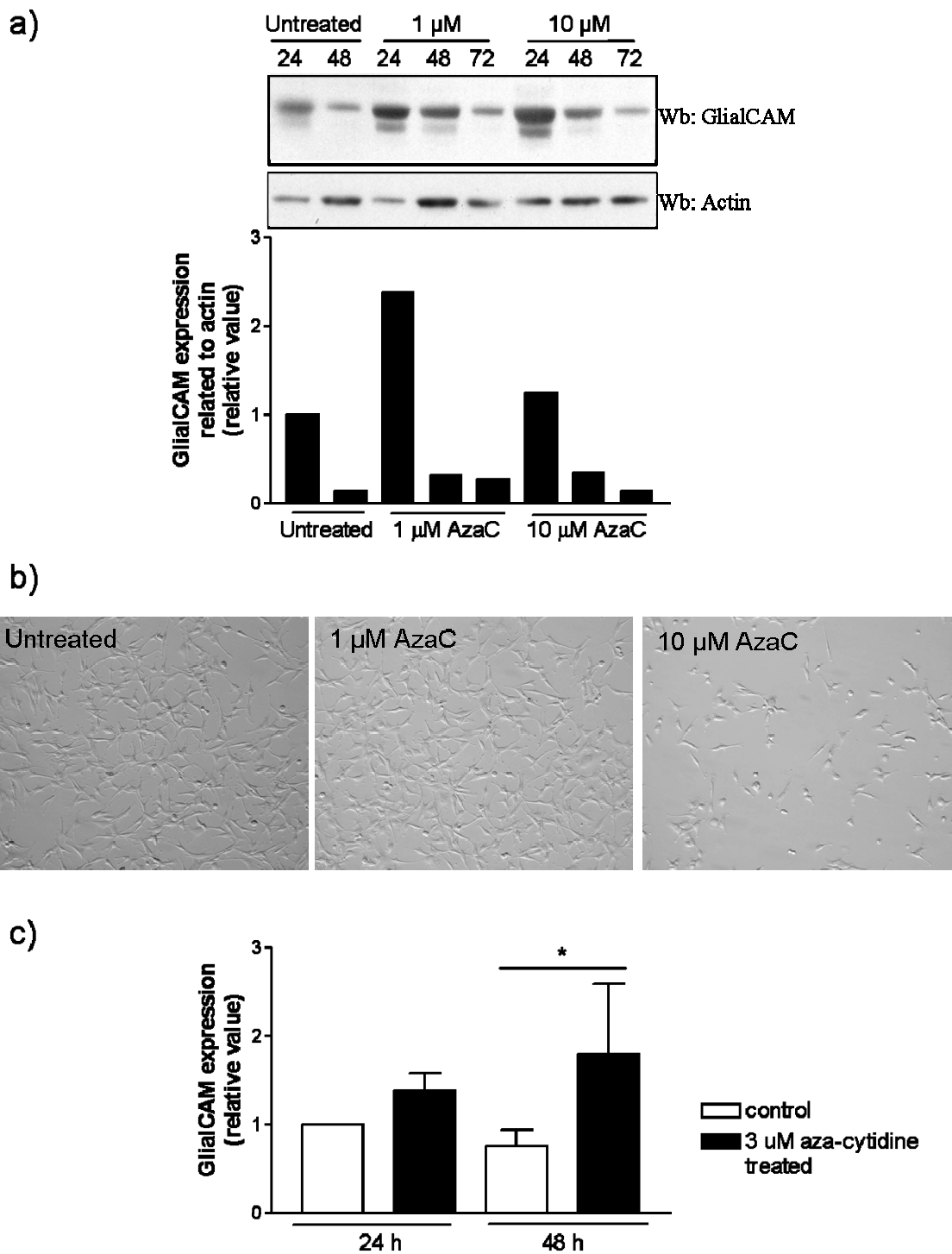


Figure 40. GlialCAM expression increases in A-172 glioblastoma cells when DNA methylation is inhibited using Aza-cytidine (AzaC). A) Western blot analysis of AzaC treated A-172 cells shows that GlialCAM expression is increased after 24 h incubation with 1 and 10 μM AzaC compared to ctrl (untreated cells). GlialCAM expression levels drop to ctrl levels within 72 h after treatment. 10 μg protein was loaded per lane. GlialCAM expression levels were normalised to the actin levels. B) 24 h treatment with 10 μM AzaC shows

clear cytotoxicity on the A172 cell, while 1 μ M treated cells look like the untreated samples. C) Western blot analysis of 3 μ M AzaC treated A-172 cells shows that GlialCAM expression is significantly increased 48 h after the beginning of treatment compared to the untreated cells at the same timepoint. The results represent triplicates. The data was analysed using Bonferroni's Multiple comparison test, where $*p < 0.05$ comparing treated to untreated cells at the same timepoint.

2.2.1.6. GlialCAM deficient mice show altered myelination compared to WT littermates

GlialCAM deficient mice show no apparent phenotypic alterations. Since the protein is highly expressed in white matter rich regions and more specifically OLS, we decided to look more closely at possible myelination defects in these mice. The work in this section was done in collaboration with Alexandre Rolland and is only a preliminary characterisation of the GlialCAM deficient mice. Brains from 4-month-old GlialCAM deficient and WT littermate mice were isolated, the RNA was extracted, transcribed to cDNA using reverse transcriptase and levels were determined by quantitative PCR (qPCR). As shown in figure 41, there is a significant decrease in MBP and CNPase levels in GlialCAM deficient mouse brains compared to controls, while no significant difference in expression levels of MOG, PLP, SNAP25 (synaptosomal-associated protein of 25 kD) or MAP2 (Microtubule-associated protein 2) were detected. Myelin related proteins MBP, CNPase, MOG and PLP have been discussed in the introduction. SNAP-25 is expressed on neuronal axons, is part of the SNARE complex and is involved in docking and fusion of vesicles with the plasma membrane (Osen-Sand, A, J Comp Neurol, 1996). SNAP-25 expression is not detected in glial cells (Hepp, R, Glia, 1999). MAP2 is a neuron-specific microtubule-associated protein, which plays a role in maintaining microtubule growth and is involved in determining and stabilizing dendritic shape during neuron development (Dehmelt L, J Neurobiol, 2004).

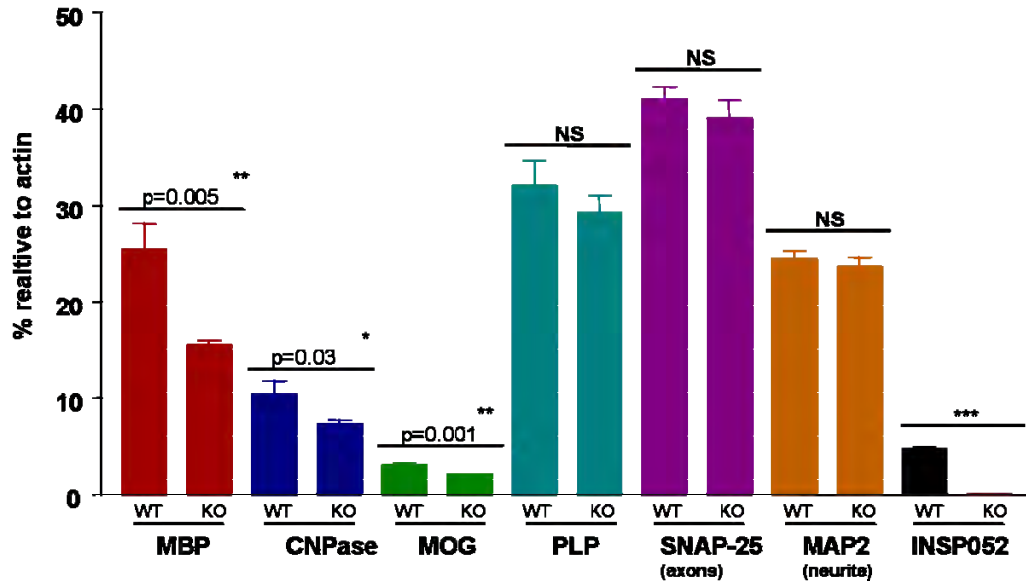
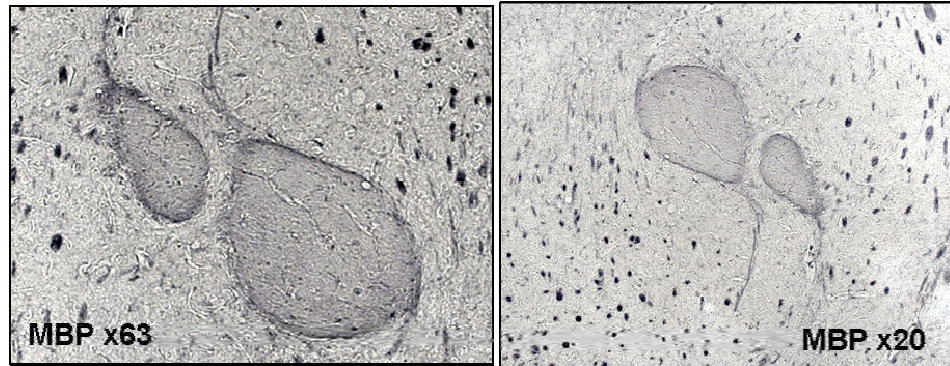


Figure 41. GlialCAM deficient mice brains express myelin related proteins at lower levels than WT littermate controls. Quantitative PCR analysis shows that GlialCAM deficient mouse brain expresses lower levels of MBP, CNPase and MOG compared to WT controls, while PLP, SNAP-25 and MAP2 expression remains the same. The data represents 4 per group. The data was analysed by unpaired t-test comparing WT to GlialCAM $-/-$ OPCs.

MBP immunohistochemistry on GlialCAM deficient versus WT littermate mice brains revealed structural defects in the myelin sheets. Figure 42 shows MBP immunostaining in the anterior commissure and interestingly vacuoles that are not present in the WT littermates can be detected in GlialCAM deficient myelin tracts. This indicates defects in the compaction of the myelin sheet and suggests a role of GlialCAM in adhesion between the extracellular membranes that form the interperiod line of the myelin sheet described in the introduction, similarly to observation made on PLP and PLP splice variant DM20 double knockout mice (Coetzee et al., 1999).

WT mice



GlialCAM $-/-$ mice

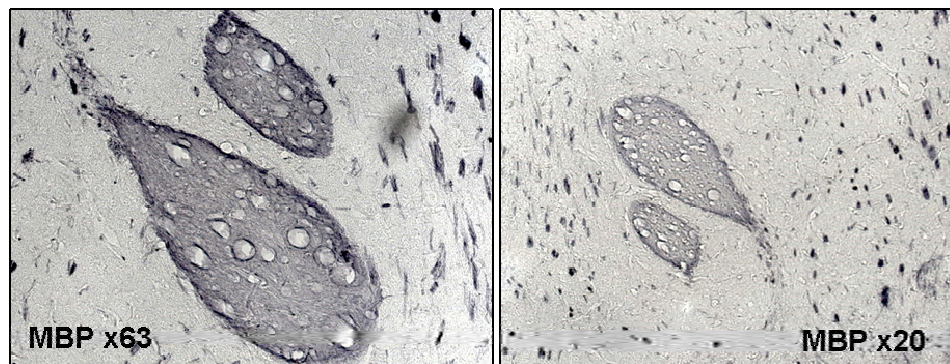


Figure 42. MBP staining shows vacuoles in the anterior commissure, indicating a defect in myelin compaction. Mouse brain sections were stained with an anti-MBP antibody.

2.2.2. Discussion

The present study shows that, in the CNS, GlialCAM is regulated through phosphorylation, glycosylation and alternative splicing. Furthermore, we demonstrate that the protein is involved in important functions relating to OLs, including OPC migration, OL myelination and OL tumorigenicity.

Glycosylation is an important regulator of IgCAM integrity and function. The carbohydrates attached to the protein backbone may regulate movement in the cell membrane thereby optimising interactions with membrane proteins on opposing cells. They may shield the protein from non-specific interactions and protect the protein from proteolytic degradation (Barclay, 2003). The results obtained in this study indicate that, in mouse brain, the carbohydrates on GlialCAM protect the protein from degradation, since deglycosylation of GlialCAM leads to rapid degradation of the protein *in vitro*. However, the other possible functions of GlialCAM associated carbohydrates mentioned above are still to be determined. We demonstrate that the two CNS isoforms of GlialCAM that were observed by Western blot are most likely not due to differential glycosylation of a single protein backbone, because deglycosylation of the protein does not render a single band, only the size of the bands were reduced. These results together with the report of two GlialCAM splice-variants in human brain suggest that the two bands that were observed in human and mouse CNS are a result of alternative splicing (Ota et al., 2004). GlialCAM splice-variants differ in the intracellular domains, indicating possible differences between the two proteins in signalling, targeting and sorting within the cell and interactions with the cytoskeleton. MAG, which is described in the introduction, also exists as two isoforms generated by alternative splicing. Interestingly, the two MAG isoforms are regulated during development much like GlialCAM splice-variants (see section on GlialCAM expression), where the large isoform is detected from early on in development correlating to myelinogenesis, while the small isoform increases with maturation and the two isoforms are present at almost equal amounts in adults (Quarles, 2007). The small and large isoforms of MAG seem to exert similar but diverging functions, because OLs that myelinate many relatively small axons express the large isoform of MAG and OLs that myelinate only a few larger axons express the small isoform. The differences in the intracellular domains of MAG isoforms leads to altered signal transduction and cellular targeting. While the MAG large isoform

binds to phospholipase C and S-100b, the small isoform binds to tubulin and zinc. Both isoforms bind Fyn, but high affinity binding via SH2 and SH3 on Fyn and subsequent activation has only been demonstrated for the larger isoform. Interestingly, the cytoplasmic tail of GlialCAM contains proline rich regions that provide putative binding sites for SH3 domains and GlialCAM co-localises with Fyn on the cell membrane (Moh M, FASEB J, 2006 meeting abstract). It could be intriguing to study whether GlialCAM binds Fyn and, in case of binding, whether binding efficiencies differ between the GlialCAM isoforms. Moreover, future studies should focus on identifying binding partners to the two GlialCAM isoforms, which could give insight into their functions.

The present data do not support the previous report that GlialCAM primarily forms cis-homo dimers, opposed to trans-homo dimers (Chung et al., 2005). In HEK293 cells, ectopic expression of GlialCAM did not induce GlialCAM cis-homo dimerisation but rather cell clustering indicating trans-homo interactions. The reason why the results differ from those that were previously published may come from the different cell types that were used and also, in this study, GlialCAM was expressed untagged, while in the previous study it was expressed as a recombinant fusion protein with a GFP or with a V5 tag. GlialCAM expressing HEK293 cells form cell clusters, probably through homo-trans interactions, because clustering is seen between GFP-labelled cells with ectopic expression of GlialCAM. Furthermore, in solution sGlialCAM mainly exists as a dimer demonstrating the GlialCAM has the potential to form homotypic interactions (data not shown). GlialCAM homotypic trans interactions could be important for interactions of cell processes within the same cell or intercellular interactions, including between OLS, astrocytes, or between OLS and astrocytes. GlialCAM might also form hetero-interactions. JAMs, which show close sequence homology with GlialCAM form both homophilic and heterophilic interactions. Heterophilic interactions include interactions with other members of the JAM family as well as interactions between JAMs and other types of cell adhesion molecules, such as integrins (Mandell and Parkos, 2005). For instance, JAM-A binds to $\alpha v \beta 3$ integrin on endothelial cell membranes and the complex is dissociated upon growth factor, e.g. bFGF, stimulation (Naik et al., 2003a). The sequence of cis and trans interactions of JAMs is still largely unknown. JAM proteins may bind homophilically in cis on the surface of endothelial or epithelial cells while simultaneously binding in trans to integrins on the surface of migrating leukocytes. Alternatively, JAM interactions may involve a series of adhesive interactions that occur sequentially. Extensive future investigations will be required in order to identify possible

heterophilic interacting partners of GlialCAM and to identify possible sequential cis and trans interactions.

The agarose drop assay measures cell migration induced by the innate motility of cells and the high concentration of cells in the drop, enhanced by the action of haptotactic (inducers of directional motility or outgrowth of cells) stimuli or growth factors (Frost et al., 2000). Both PDGF-AA and bFGF induce migration of rat OPCs in this assay, however the former is much more effective than the latter (Simpson and Armstrong, 1999). These growth factors can also act as chemotactic agents for OPC migration in chemotaxis assays (Zhang et al., 2004). Our results demonstrate that sGlialCAM together with PDGF-AA enhances OPC migration in the agarose drop assay. When using an OPC concentration in the drop showing migration in the presence of PDGF-AA, like reported by others, we do not see a distinct difference in migration with or without the presence of sGlialCAM. However, when using lower concentrations, where PDGF-AA alone does not induce migration, the addition of sGlialCAM together with PDGF-AA triggers OPC migration from the drop. This migration promoting effect is not due to sGlialCAM enhancing OPC proliferation, as shown experimentally. The influence of the cell concentration in the drop on the migration could be due to properties similar to endothelial cells, where the response to VEGF (vascular endothelial growth factor) is cell density-dependent (Dejana, 2004). OL morphology, and hence the panel of proteins expressed at the cell surface, has been demonstrated to be directly dependent on cell density, which could mean that PDGF and sGlialCAM response might depend on cell density (Hugnot et al., 2001). However, it cannot be excluded that sGlialCAM has an enhancing effect on OPC migration also at the higher cell concentration but that the effect is masked because of the strong migratory response created by PDGF. For example, bFGF and PDGF-AA each activates OPC migration alone, while no significant additive effect on migration is seen when treating the cells with both growth factors simultaneously (Simpson and Armstrong, 1999).

PDGF-AA acts as a disulfide-linked covalent homodimer that induces cell surface receptor tyrosine kinase PDGFRA dimerisation and activation (Heidaran et al., 1993). This leads to a rapid and robust tyrosine phosphorylation of PDGFRA, inducing several downstream signalling cascades including PI₃K, MAPK and PLC γ pathways. A recent study shows that ERK2 (p42^{MAPK}) and ERK1 (p44^{MAPK}) activation is required for PDGF-AA induced OPC migration in the agarose drop migration assay, while PI3K and p38^{MAPK}

are not essential (Frost et al., 2008). PDGFRA activates ERK2 at higher levels than ERK1 (Yim et al., 2001). Interestingly, we found that the GlialCAM intracellular domain can be phosphorylated *in vitro* by ERK2, but not $PI_3K\gamma$. This raises the intriguing hypothesis that PDGF-AA induced activation of ERK2 subsequently leads to the activation of GlialCAM, which then participates in the intricate protein network to induce OPC migration (see figure 43, black pathway). Since PDGF acts as a chemoattractant for OPC migration *in vivo*, leading to their migration from the germinal zones to the white matter tracts, GlialCAM could be involved in related cell differentiation or adhesion. Future investigations should determine whether ERK2 phosphorylates GlialCAM in OPCs and whether the phosphorylation is important for sGlialCAM induced OPC migration.

Ectopic expression of L1 (described in the introduction) combined with PDGF-AA induced PDGFR stimulation induces sustained ERK activation and cell motility (Silletti et al., 2004). Several components of the MAP kinase cascade phosphorylate directly serines in the L1-CAM-cytoplasmic domain. These include two serines that are direct targets for ERK2 phosphorylation (Schaefer A jbc 1999). Interestingly, L1-mediated ERK activation was found to be critically dependent upon cell density, however in the case of L1 high cell density led to a response while low density did not. The motile response is mediated by integrins, however whether it is through direct binding of L1 with the integrin is still unclear (Maness and Schachner, 2007). It is not known either whether L1 modulates integrin dependent cell adhesion through increased avidity or affinity for ligands, cis dimerisation or intracellular signals. It could also regulate the motile response by co-internalisation with integrins and recycling to the leading edge of the cell. PDGFR associates with integrins upon PDGF stimulation (Schneller et al., 1997) and the association seems to be lipid raft dependent (Baron et al., 2003). It should be investigated whether overexpression of GlialCAM together with PDGF-AA mediated activation of PDGFRA enhances ERK2 phosphorylation compared to PDGF-AA stimuli alone (see figure 43, green pathway). Furthermore, it should be interesting to study whether there is a direct interaction between PDGFR and GlialCAM. Interestingly, L1 ectodomain is cleaved in mouse brain *in vivo* in a developmentally regulated manner and L1 ectodomain cleavage enhances neuronal adhesion and migration, and neurite outgrowth (Mechtersheimer et al., 2001; Maretzky et al., 2005). The ectodomain is cleaved by metalloproteases, ADAM10 and ADAM17, following different activation pathways, possibly involving signals from extracellular matrix components. L1

ectodomain induces cell migration by binding and signalling through integrins, such as $\alpha v \beta 5$ integrin, as well as through an autocrine loop (Mechtersheimer et al., 2001). The pro-migratory effect of GlialCAM/sGlialCAM could be similar to L1, where GlialCAM could function in conjunction with PDGFR and sGlialCAM induce integrin activation in OLs (see figure 43, red pathway). Furthermore, it would be intriguing to determine whether sGlialCAM interacts with integrins to promote OPC migration in the agarose drop migration assay. We have not detected a lower molecular weight protein that could represent sGlialCAM, or the transmembrane and intracellular fraction that remain after cleavage, in our expression studies, but an in-depth analysis should be performed to investigate the possible shedding of GlialCAM ectodomain. Ectodomain cleavage of membrane proteins is accelerated during pathological conditions such as inflammation and apoptosis (Hooper et al., 1997), hence suggesting that GlialCAM ectodomain shedding should be investigated under these conditions.

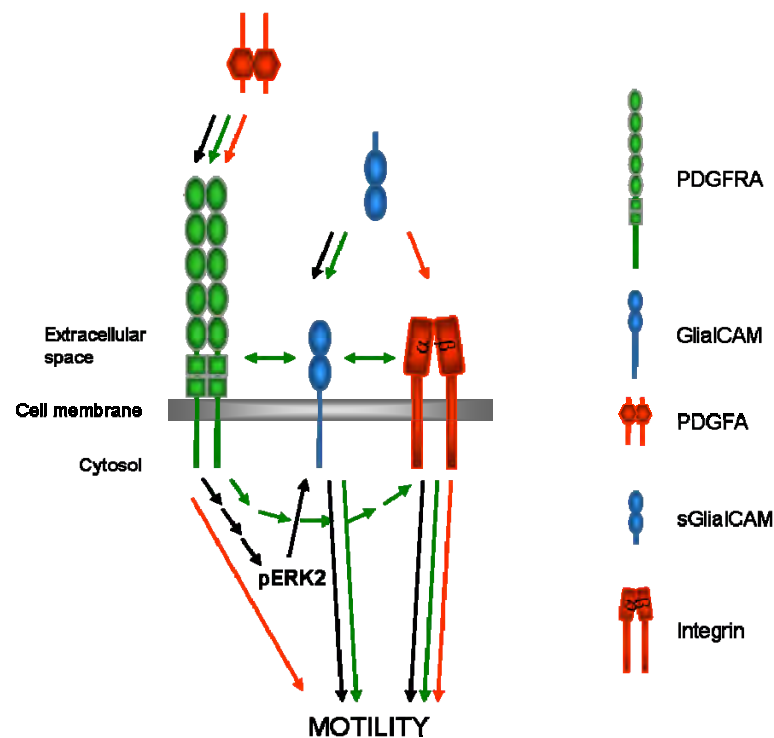


Figure 43. Schematic presentation of possible pathways through which sGlialCAM in conjunction with PDGF-AA could induce migration of OPCs. Pathway with black arrows: PDGFRA is activated by PDGF-AA and induces ERK2 activation which then activates GlialCAM, rendering a morphological change that allows sGlialCAM to bind to GlialCAM, which then activates a pathway leading to cell motility through integrins. Pathway with green arrows: PDGF-AA induces cell motility in association with integrins and sGlialCAM

induces GlialCAM to activate a pathway promoting cell motility. GlialCAM could also interact directly with PDGFR or integrins and association or dissociate for activation of motility. Also, GlialCAM bound integrin could be targeted to the leading edge through selective endocytosis. Pathway with red arrows: sGlialCAM could bind directly to integrins and activate a parallel signalling pathway to activated PDGFR to promote motility.

It should also be considered that GlialCAM might enhance OPC cell survival. The IgCAM member, Nectin-3, interacts directly with PDGFR and plays a key role in PDGF induced mouse embryonic cell survival through activation of the PI₃K-Akt signalling pathway (Kanzaki et al., 2008). Afadin binding to Nectin-3 intracellular portion is important for this response, maybe through binding of Afadin to a subunit of PI₃K, p85, that interacts with PDGFR. Whether sGlialCAM enhances OPC cell survival could be measured by labelling cells with propidium iodine and a fluorescently labelled antibody against caspase-3 to monitor for apoptotic cell death (Frost et al., 2008).

Interaction between OLs is difficult to demonstrate *in vivo* but *in vitro* studies have shown such interactions. When OLs come into contact on the cell culture dish, their growth cone-like structures, which form the original point of cell-cell contact, undergo dramatic changes in morphology (Moorman, 1996). The fine filopodia and lamellipodia of these structures are retracted, eventually leading to growth cone collapse and inhibition of OL motility. This suggests that OLs interact and respond to cell surface receptors on other OLs. GlialCAM is expressed in OL growth-cone like structure *in vitro* (see section on GlialCAM expression) and forms trans interactions, suggesting that it could be involved in interactions between OLs. If this is the case, adding sGlialCAM could disrupt OPC interactions in the agarose drop migration, leading to a change in OPC morphology that allows PDGF to induce motility (see figure 44). This hypothesis is supported by migration studies of GlialCAM deficient mouse OPCs, where following FGF stimulation, GlialCAM deficient cells migrate further from the agarose drop than OPCs from WT littermates. Decreased adhesive contact between GlialCAM deficient OPCs could explain this effect. The reason why we do not see a difference in migration levels between GlialCAM deficient and WT OPCs when a higher concentration of cells is added to the drop could be because the migratory effect is already so pronounced with WT OPCs that less contact inhibition due to GlialCAM deficiency is not detected. Further studies should be performed to determine whether there are increased amounts or a different distribution of OPCs in GlialCAM deficient versus WT mice CNS. However,

higher levels of OPC migration in the absence of GlialCAM could also be due to enhanced proliferation or survival and should be assessed. Unfortunately, we were not able to study the effect of PDGF induced migration on mouse OPCs, because murine cells, unlike rat cells, do not respond to PDGF in this assay. It would have been interesting to see whether the concomitant pro-migratory effect observed with PDGF and sGlialCAM stimulation of rat OPCs requires membrane bound GlialCAM. The reason why PDGF did not induce mouse OPC migration in the assay could be because the OPCs were amplified in the presence of PDGF and bFGF after isolation and it has been shown that PDGF induces its effect only for a limited amount of OPC divisions, while OPC stimulation by bFGF is indefinite (Armstrong et al., 1990). The data on GlialCAM deficient OPCs demonstrates that GlialCAM is not indispensable for OPC migration and only subtle myelin defects have been observed in knockout mice CNS. However, the lack of GlialCAM in these mice might be compensated by other protein pathways. Similarly, OSP/Claudin-11 is not indispensable for OPC migration in the agarose drop migration assay, and the knockout mice have only subtle myelin defects, but OSP/Claudin-11 deficient OPCs show reduced migration on fibronectin in the agarose drop migration assay. It could be interesting to examine GlialCAM deficient OPC migration on different substrates, such as vitronectin, fibronectin and laminin, to study if GlialCAM has a co-operative pro-migratory function with the integrins, $\alpha 6 \beta 1$, $\alpha v \beta 1$, $\alpha v \beta 3$, that are expressed in OPCs *in vitro* (Tiwari-Woodruff et al., 2001). If differences in migratory properties on specific substances were seen compared to WT OPCs, it would support a migratory opposed to a “de-clustering” effect of GlialCAM.

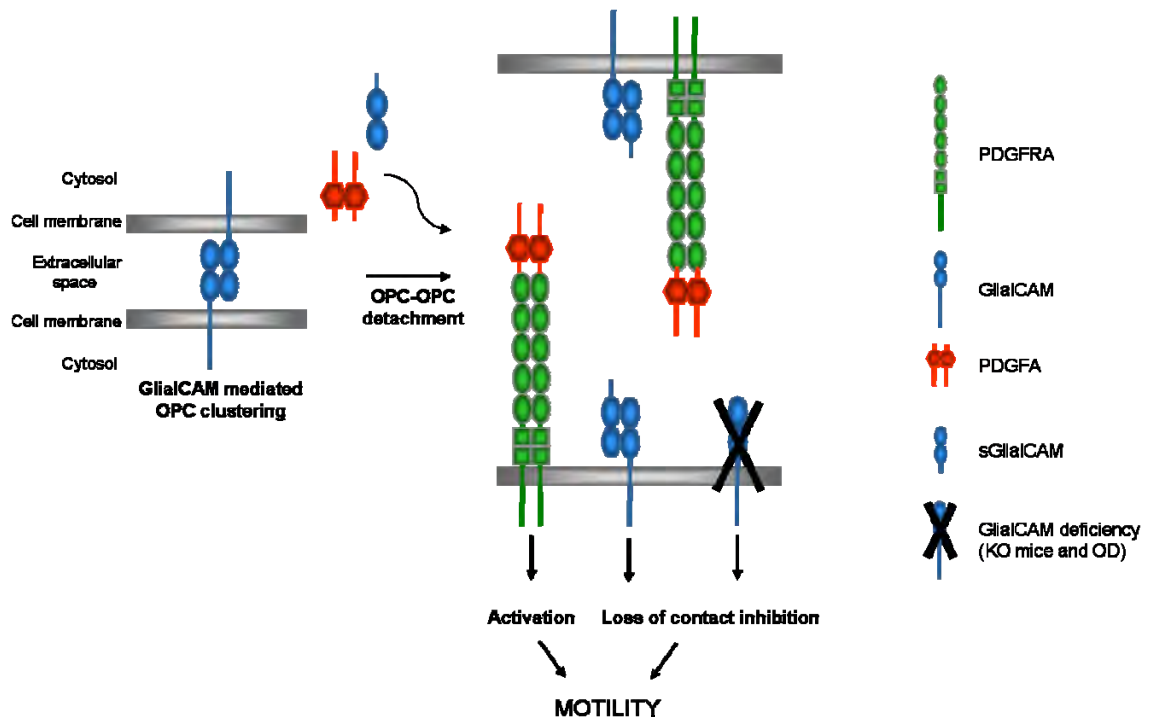


Figure 44. Schematic presentation of sGlialCAM or GlialCAM deficiency induced loss of OPC contact inhibition. OPCs form cell-cell contacts that could be in part through GlialCAM homotypic interactions. When sGlialCAM is added to the culture medium it possibly dissociates these contacts through competitive binding and PDGF-AA induces cell motility. OPCs lacking GlialCAM, in the GlialCAM knockout (KO) mice or in oligodendroglioma (OD) grade III, could similarly have less contact inhibition and hence increased motility compared to WT controls.

Downregulation of IgCAMs, including NCAM and CAR, in gliomas leads to enhanced tumorigenicity and cell invasion, as described in the introduction. The molecular mechanisms by which these proteins enhance tumour invasion is still under study, but current data indicate that loss of NCAM results in defective cell-matrix adhesion which leads to cell detachment and invasion, while loss of CAR leads to decreased microtubule stability and a transition from a quiescent to a invasive, mesenchymal phenotype. In other cases, loss of CAM mediated cell-cell adhesion is a prerequisite for tumour invasion, such as for E-cadherin (Cavallaro and Christofori, 2004). We have shown in the present study that GlialCAM is downregulated in human grade II and III oligodendrogliomas and grade I and IV astrocytomas. Combining the data demonstrating that 1) GlialCAM is downregulated in higher grade oligodendrogliomas, which have increased invasive properties compared to lower grade, and 2) that GlialCAM deficient OPCs show enhanced migration and finally, 3) that sGlialCAM enhances OPC migration,

would suggest that GlialCAM is involved in cell adhesion between OLs (see figure 44). In this case loss of GlialCAM in gliomas would correlate with loss of cell-cell contact and thereby lead to enhanced tumorigenicity and invasion, as for E-cadherin. However, further studies are required to determine the role of GlialCAM in tumorigenicity.

Methylation is the most common epigenetic variation in human cancers (Kanai and Hirohashi, 2007). Several tumour cells have been shown to have genome wide hypomethylation with regional hypermethylation at the 5-carbon position of cytosine, especially at the promotor CpG-islands of a variety of tumour related genes. Tumour suppressor genes are commonly silenced through hypermethylation. Examples of CAMs that are silenced through hypermethylation in human cancers, include E-cadherin and NeCl-2 (Yoshiura et al., 1995; Heller et al., 2006). The IgCAM, NeCl-2 is involved in cell-cell adhesion, cell motility and apoptosis, and loss of the protein in tumours leads to impaired cell adhesion between tumour cells and to metastasis. Interestingly, when the A-172 human glioblastoma cell line is incubated with an inhibitor of DNA methylation, 5-azacytidine, we observed enhanced levels of GlialCAM expression, indicating that the expression of the protein is downregulated in glioma cells through hypermethylation. Hypermethylation of GlialCAM could be used as a marker for glioma tumorigenicity, as has been suggested for other genes (Kanai and Hirohashi, 2007). Furthermore, GlialCAM gene-specific demethylation agents could be of therapeutic value in the treatment of gliomas. Moreover, the Raf-MEK-ERK pathway is frequently up-regulated in cancer cells and is known to down-regulate cell-cell adhesion molecules. For instance, CAR is downregulated through the Raf-MEK-ERK signaling pathway in a variety of cancer cell lines and inhibitors of MEK activity enhance CAR expression, suggesting a potential for developing cancer therapies (Anders et al., 2003). Since GlialCAM intracellular domain is phosphorylated *in vitro* by ERK2, it could be interesting to study whether MEK inhibitors alter GlialCAM expression in gliomas.

As described in the introduction, there is a lack of reliable markers for gliomas, especially oligodendrogliomas (ODs). The most robust objective marker for OD today is the allelic loss of the 1p and 19q loci, while histological analysis of tumour samples are subjective to the investigator. Together with a panel of other markers, changes in GlialCAM expression could be used to identify gliomas. Furthermore, the relevant decrease in GlialCAM expression in grade III versus grade II ODs could be a valuable therapeutic marker. Grade III OD cells have an altered phenotype, including increased

mitotic activity, microvascular proliferation, motility, and/or spontaneous necrosis, and patients have decreased median survival time compared to grade II OD. These properties are relevant when designing appropriate therapies.

The present study demonstrates for the first time that GlialCAM is phosphorylated, not only following ectopic expression in HEK cells but also in primary rat astrocytes. Furthermore, *in vitro* studies show that the purified intracellular portion of the protein can be phosphorylated by ERK2 and ASK1. Phosphorylation is probably an important feature for the regulation of GlialCAM function and the intracellular domain contains several potential phosphorylation sites. Possible roles of ERK2 regulated phosphorylation of GlialCAM have been suggested above. ASK1 is a intracellular serine/threonine mitogen-activated protein (MAP) kinase kinase kinase that was first identified as a mediator of TNF α -induced apoptosis by activation of the p38 MAP kinase and the c-jun N-terminal-activating kinase pathways (Ichijo et al., 1997). However, in some cell types it promotes cell survival and differentiation rather than apoptosis, suggesting that it might have opposing functions depending on the cell type and state. In adult neural progenitor cells that have the potential to give rise to neurons, astrocytes and OLs, ectopic expression of ASK1 promotes neuronal differentiation, while glial cell development is inhibited (Faigle et al., 2004). Taking into account that ASK1 inhibits glial differentiation and that GlialCAM is expressed in all stages of OL development and in astrocytes, one could hypothesise that GlialCAM phosphorylation by ASK1 has an inhibitory effect on GlialCAM function.

Preliminary studies on CNS myelination in GlialCAM deficient mice show reduced MBP and CNPase levels, and vacuoles in the anterior commissure white matter tracts. This could suggest a role for GlialCAM in myelin tight junctions, since similar myelin abnormalities have been observed in double knockout mice for PLP and OSP/Claudin-11 or GalC/sulphatide (Chow et al., 2005; Coetzee et al., 1999). In addition, in mice deficient for the large MAG isoform the most common myelin defect was the retention of cytoplasm within the myelin lamella, suggesting a delayed compaction process (Fujita et al., 1998). In addition to the hypothesis above that GlialCAM might form homo-interactions between opposing OPCs, it might also form adhesive interactions within the myelin sheet. However, further studies are required to elucidate this point.

Remyelination is a common feature of early stage multiple sclerosis (MS), however with repeated or prolonged episodes of demyelination during the course of the disease,

the inherent capacity of the CNS to remyelinate becomes exhausted and the axon remains demyelinated and becomes vulnerable to atrophy. OPCs in the adult CNS are believed to be the major source of remyelinating OLs (Levine et al., 2001). In animal models of demyelination, one strategy to promote the repair of chronically demyelinated lesions would be to enhance or reactivate OPC mediated remyelination. Local administration of PDGF-AA enhances remyelination of lysophosphatidylcholine induced demyelinated lesions in the corpus callosum in rats (Allamargot et al., 2001). Furthermore, remyelination of the corpus callosum following chronic demyelination induced by cuprizone treatment was significantly improved in transgenic mice that overexpress PDGF-AA in astrocytes under the control of GFAP gene promoter compared to WT controls (Vana et al., 2007). The mechanisms by which PDGF-AA improves remyelination could include OPC proliferation, survival and migration. Taken the pro-migratory effect on OPCs of sGlialCAM in conjunction with PDGF-AA and the need for OPC migration to the site of myelin damage in demyelinating diseases, such as MS, it could be interesting to study the effect of sGlialCAM with PDGF-AA on CNS remyelination. As a primary investigation, this could be done by stereotaxic microinjection into rats (Allamargot et al., 2001). Indeed, L1 ectodomain, which could have similar functions as sGlialCAM, however in neurons, is suggested as a potential pharmaceutical to promote nerve regeneration (Sugawa et al., 1997).

Taken together, we demonstrate for the first time that GlialCAM is glycosylated in mouse brain and that deglycosylation of the protein leads to its rapid degradation, that it is phosphorylated in astrocytes *in vitro* and that ERK2 and ASK1 phosphorylates the intracellular domain *in vitro*. Furthermore, we do not detect GlialCAM cis-dimerisation, while the protein forms trans-interactions. sGlialCAM enhances OPC migration and GlialCAM deficient OPCs show enhanced migration compared to WT controls. Furthermore, GlialCAM is downregulated in oligodendrogliomas and astrocytomas, most likely through hypermethylation. These findings provide a basis for the intriguing challenge of future investigations to find out the role of GlialCAM.

2.2.3. Materials and methods

2.2.3.1. Plasmid constructs

All the constructs were subcloned with the Gateway™ technology (Invitrogen). This technology is a universal cloning method based on the site-specific recombination properties of bacteriophages lambda, where lambda facilitates the integration of the DNA into the E. Coli chromosome and the switch between the lytic and the lysogenic pathways. The technology provides a rapid and efficient way to move DNA sequences into multiple vector systems for functional analysis and protein expression (Hartley et al., 2000). We largely followed the manufacturer's protocol for cloning with the Gateway™ technology, as described below.

The cDNA of the recombinant fusion protein containing a N-terminal β 2-microglobulin followed by a caspase-8 cleavage site, 6-his tag and the intracellular domain of GlialCAM (GlialCAM-ICD) was cloned into the pEAK12d expression vector. A signal sequence for protein secretion was added at the 3' end of the open reading frame. First, the β 2-microglobulin cDNA was amplified by PCR from a donor plasmid using a forward primer containing the kozak sequence and *attB1* sequence for gateway cloning, and a reverse primer containing part of the caspase-8 cleavage site sequence. GlialCAM-ICD was amplified by PCR from a donor plasmid using a forward primer containing part of the caspase-8 cleavage site sequence, and a reverse primer containing *attB2* sequence for gateway cloning. The samples were separated on a 1.5 % agarose gel and the DNA visualised using ethidium bromide. The bands corresponding to the desired DNA fragments were cut from the gel and the DNA was extracted using the Wizard® SV gel and PCR clean-up system (Promega) and eluted into nuclease-free water. A second round of PCR amplification was performed using primers that overlap at the caspase-8 cleavage site and 6-his tag sequence at the 3' end of the β 2-microglobulin cDNA and 5' end of the GlialCAM-ICD cDNA, respectively. The PCR products were again separated on an agarose gel and extracted as above. A final PCR amplification was made to combine the β 2-microglobulin GlialCAM-ICD cDNA containing open reading frames into a single fragment that was again isolated through a gel extraction. The linear fragment was subcloned into the pDONR221 (figure 45) donor plasmid using *attB* and *attP* recombination and then further through *attL* and *attR* recombination into the pEAK12d (figure) mammalian expression vector.

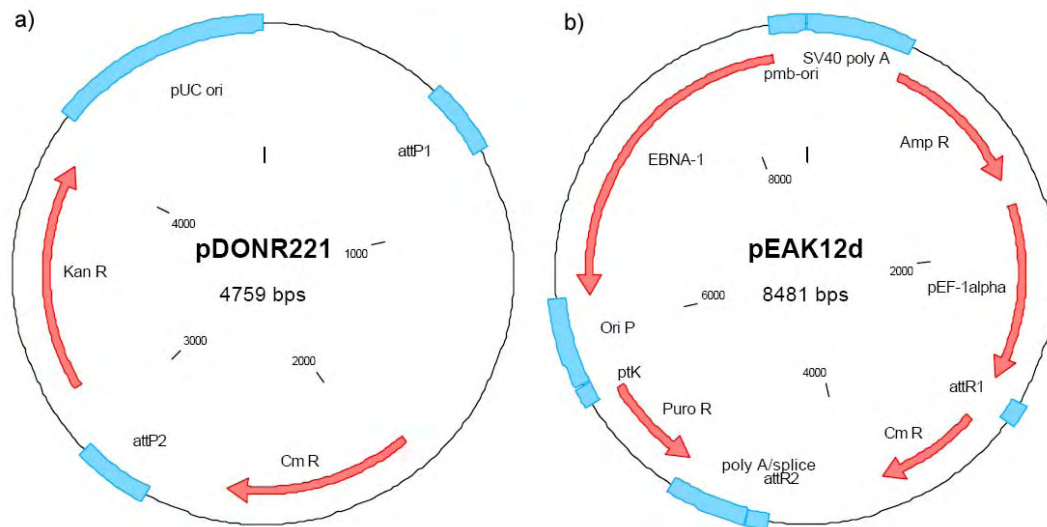


Figure 45. Map of the gateway entry vector pDONR221 (a) and the mammalian expression vector pEAK12d (b).

NeCl-1 and CD147 were amplified by PCR from image clones using primers containing the *attB* sites for gateway recombination and subcloned first into the pDONR221 and then the pEAK12d expression vector, largely as described above. The cDNA of GlialCAM, CD147 and EGFR were amplified by PCR from plasmids using primers containing the *attB* sites for gateway recombination and subcloned first into the pDONR221 and then into a pEAK12d expression vector that had been modified so that the resistance gene to the Puromycin was substituted by eGFP gene marker.

2.2.3.2. Transfecting adherent and suspension HEK cells

700 000 adherent HEK293 cells were seeded in 2 ml medium (DMEM medium (4500 mg/L D-glucose, Invitrogen) supplemented with 10% Foetal Calf Serum (FCS, Sigma), 2 mM L-glutamine (Invitrogen), 100 U/ml penicillin and 100 µg/ml streptomycin (Sigma) per 6 well plate well, 24 h before transfection. The cells were transfected with lipofectamine 2000 (Invitrogen) transfection reagent largely following the instructions of the manufacturer. The lipofectamine reagent was diluted 1:30 in Opti-MEM reduced serum medium (Invitrogen) and further 1:1 with 10 µg/ml DNA in Opti-MEM. The DNA-transfection agent complex was mixed and incubated at room temperature for 20 min

and then added to the cells in a drop-wise manner. 80 to 90% cells confluency is needed to maximize the transfection efficiency. The cells were incubated at 37°C in 0.5% CO₂ for 24, 48 or 72 h. Transfection efficiency and expression was analysed by fluorescence microscopy (eGFP fluorescence) and SDS-PAGE electrophoresis combined with Western blot, as described above (Favre-Kontula, L. et al. *Glia* 2008). For Western blot analysis, polyclonal anti-EGFR (Fitzgerald) and polyclonal NeCl-1 antibody (kind gift from Takai, Y) (Kakunaga et al., 2005) were diluted 1/1000 in TBS containing 0.1% Tween-20 and 5% skimmed milk and the nitrocellulose membrane was incubated for 1 h at RT with the antibody solution.

HEK/EBNA suspension cells were grown in 500 ml spinners in ExCell-Vpro medium (Sigma) supplemented with 4 mM L-glutamine and 1 ml/l of a 0.5 % (w/v) phenol red solution. The cells were harvested by centrifugation at 250 x g and resuspended at a concentration of 1×10^5 cells/ml in 200 ml DMEM/F12 medium supplemented with 29 mM NaHCO₃, 10 mM Hepes, 5 g/l D-glucose, 7.5 mM L-glutamine, 1 % FBS and 4 ml/l ITSx (Insulin, 1 g/l; sodium selenite, 0.67 mg/l; transferrin, 0.55 g/l; and ethanolamine, 0.2 g/l) (Invitrogen). A transfection mix containing 500 mg DNA and 1 mg polyethylenimine (PEI) (Polysciences) was prepared in 50 ml of the medium above by vortexing the solution and incubating at RT for 10 min. The transfection mix was added to the cells in a 500 ml spinner flask and incubated for 90 min in a humidified incubator containing an atmosphere of 5 % CO₂. 250 ml of Freestyle medium (Invitrogen) was added to have a final volume of 500 ml and the culture maintained in serum-free medium for 6 days.

2.2.3.3. Purification of soluble his-tagged proteins

HEK/EBNA suspension cells were transfected with the pEAK12d plasmid containing the cDNA encoding for the β 2-microglobulin-GlialCAM-ICD fusion protein, the cell supernatant was harvested and several purification and cleavage steps were conducted to isolate the 6-his tagged GlialCAM-ICD. The cell culture supernatant (450 ml) from HEK/EBNA cells was harvested 6 days after transfection by centrifugation the supernatant for 15 min at 230 x g. The supernatant was diluted with 2 volumes of equilibration buffer (50 mM NaPO₄ buffer, pH 7.5, containing 0.6 M NaCl and 8.7 % (v/v) glycerol) and the sample was filtered through a 0.22 mm filter membrane. The sample solution was loaded on a Porous 20 MC (Applied biosystems) charged with Ni²⁺ ions and equilibrated in the equilibration buffer using a Vision purifier system (Applied

biosystems). Nonspecifically bound material was removed by washing the column with equilibration buffer and bound proteins were eluted by a linear gradient of 12.5 to 250 mM imidazole in equilibration buffer. According to the elution profile determined by absorbance at 280 nm, the protein containing fractions were pooled. The pool was concentrated 10-fold using centrifugal filter devices with a cut-off of 10 kDa (Amicon Ultra-15). The concentrated pool was subjected to size exclusion chromatography as a second step of purification. 15 ml of the concentrated pool was loaded onto a Superdex 200 column (GE healthcare) with a bed volume of 330 ml, equilibrated in PBS. The protein was eluted and according to the elution profile, fractions were selected and analyzed by SDS. Fractions containing the protein of interest were pooled together, concentrated as above, 20 µg Caspase-8 was added per mg protein and the solution was incubated for 2 h at RT. The protein solution was then again subjected to metal affinity chromatography as described above, in order to remove Caspase-8 and β 2-microglobulin that is cleaved from the fusion protein. GlialCAM-ICD was eluted with Imidazol and the eluted fractions were concentrated as above and 30 ml was loaded on a coarse G-25 size exclusion column equilibrated in 20 mM Tris pH8.5 (at 4°C) to change the buffer. The solution was then loaded on a SP sepharose cation exchange column (Amersham biosciences) using the Äkta purifier system in order to remove any uncleaved protein. A cation exchange column and 20 mM Tris pH 8.5 buffer were chosen for this step based on the charge difference of the 6His tagged GlialCAM-ICD (charge = 6.55 at pH 8.5) and β 2-microglobulin-GlialCAM-ICD fusion protein (charge =1.30 at pH 8.5). Fractions containing the cleaved protein were pooled and the buffer was changed using a coarse G-25 size exclusion column equilibrated with 20 mM Tris pH7.5 (at 4°C). The protein solution was concentrated as above and stored at -80°C.

The other his tagged proteins, sCD147 and sGlialCAM, were purified using the same procedure, Ni^{2+} -NTA metal affinity column followed by SEC on a Superdex 200 column.

2.2.3.4. Mouse brain protein extraction

Adult female C57B6 mice were anaesthetized with pentobarbital (50 mg/kg i.p.) and then perfused through the left cardiac ventricle with PBS containing 1 mM EDTA. The brains were extracted and placed in eppendorff tubes containing cold triple detergent buffer (50 mM Tris, pH 8.0 containing 150 mM NaCl, 0.02% NaN_3 , 0.1% SDS, 1% Nonidet P-40, 0.5% sodium deoxycholate) and a Complete EDTA-free protease inhibitor

cocktail (Roche Molecular Biochemicals, 1 tablet per 50 ml buffer). The samples were homogenised on ice using a polytron, then centrifuged at 89 000 x g (50 000 rpm in the TLA120.2 rotor (Beckman Instruments) for 20 min and the supernatant containing the soluble proteins was collected. Protein concentration was measured using a BCA (bicinchoninic acid) - protein assay kit (Pierce).

2.2.3.5. Protein deglycosylation

Mouse brain protein extract or purified 6-his tagged sGlialCAM were treated with deglycosylating enzymes according to the manufacturer's protocol for the Enzymatic CarboRelease™ kit (QA-Bio). 4 mg/ml of brain extract or 40 µg/ml of sGlialCAM was incubated with 2.7 mU sialidase Au, 2.7 mU PNGase F, 0.7 mU O-glycosidase, 27 mU β-galactosidase and 0.5 mU glucosaminidase for 0, 4, 8 and 24 h at 37 °C. Incubations with the glycosylation enzymes were carried out under denaturing conditions in the denaturation solution provided by the manufacturer and in the presence of Triton X-100. SDS sample buffer (1:5) (0.125 M Tris, pH 6.8; 4% (w/v) sodium dodecyl sulfate (SDS); 20% (v/v) glycerol; 0.2 M DTT; 0.02 % (w/v) bromophenol blue; 1% (w/v) Na-deoxycholate; 2% (v/v) Triton X-100) was added to the samples after the respective incubation times. The samples were analysed by Western blot using the anti-GlialCAM polyclonal antibody as described above (Favre-Kontula, L et al. *Glia*, 2008).

2.2.3.6. Immunoprecipitation

For immunoprecipitation, cells were homogenised and membrane proteins extracted using cold lysis buffer (20 mM Tris, pH 7.4 containing 1 mM Na₃VO₄, 25 mM β-glycerophosphate, 10 mM NaF, 1 mM Na-Pyrophosphate, 250 nM okadaic acid, 10 nM calyculin, 150 mM NaCl, 1 % Triton X-100, 0.5 % sodium deoxycholate). 0.5 ml lysate at 1 mg/ml was incubated for 1 h on a roller at 4°C together with 50 µl of Ultralink Immobilized Protein A/G Plus Sepharose beads (Pierce) to reduce unspecific binding. The beads were removed by centrifugation and the pre-cleared lysate was incubated with 2 µg of specific antibody overnight at 4°C. 20 µl of Sepharose beads were added to the antibody-lysate mixture, which was further incubated for 1 h at 4°C on a roller. The beads were washed three times with complete lysis buffer and beads were resuspended in 50 µl of 5X SDS sample buffer and incubated for 5 min at 95°C. The proteins from the

immuno-complex were separated on a 4-12 % gradient NUPAGE Bis Tris gel (Invitrogen) and GlialCAM was detected with an anti-GlialCAM antibody.

2.2.3.7.[³³P]-orthophosphate metabolic labelling of cells

Primary rat astrocytes were isolated as described above (Favre-Kontula, L et al. *Glia*, 2008) and the P1 cells were plated at 600 000 cells per well in a 6-well plate 3 days before the experiment. HEK 293 cells were transfected for 48 h following the protocol above. Cells were at 70 to 80 % confluency at the time of the experiment. They were washed twice with phosphate-free RPMI-1640 medium (ATCC) supplemented with 10 % phosphate free horse serum and incubated for 1 h in the same medium. The cells were then labelled for 3 h with [³³P]-orthophosphate at a final concentration of 1 mCi/ml in the culture medium. After the labelling, the cells were lysed and immunoprecipitation was carried out as described above. The immuno-complex was separated on a 4-12 % gradient NUPAGE Bis Tris gel (Invitrogen), electrotransferred to a 0.45 µm nitrocellulose membrane (Invitrogen) and the membrane was exposed to a high performance chemiluminescence film (Amersham) in order to detect [³³P]-orthophosphate radiation. The membrane was then Western blotted with the anti-GlialCAM antibody to identify GlialCAM on the blot.

2.2.3.8.In vitro kinase assay

Purified recombinant kinases at a final concentration of 33 ng/ul were mixed with 330 ng/ml MBP or GlialCAM-ICD-6His in a total volume of 30 µl in kinase buffer (60 mM HEPES, pH7.4 containing 60 mM β-glycerophosphate, 6 mM DTT, 2 mM MgCl₂, 2 mM MnCl₂, 200 mM Na₃VO₄, 3 mM EDTA, 10 Ci [³³Pγ]-ATP and 0.04 mM ATP) and incubated for 1 h at 37°C. The kinases, ERK2, JNK3, MEK, CK2α, ASK1 and PI3Kγ, that were used for this assay were expressed and purified in-house. At the end of the incubation time the reaction mixtures were directly mixed with SDS sample buffer, the proteins separated on a 10 % NUPAGE Bis Tris gel (Invitrogen) and ³³P radiation was determined by exposing the gel for 1 h on a PhosphorImager screen that was further exposed on a MP hyperfilm (Amersham) overnight at -80°C.

2.2.3.9.Cis-dimer formation assay

HEK293 cells were transfected and incubated for 48 h as described above. They were washed with PBS, then incubated with 0.2% trypsin and 1 mM EDTA at 37°C for 5 minutes and dispersed by gentle pipetting. Cells were washed with PBS, resuspended at a concentration of 1×10^6 cells/ml in PBS containing 1 mM bis (sulfosuccinimidyl) suberate (BS3) crosslinker (Pierce) and 1 ml of the cell suspension was added per well in a 24 well plate coated with BSA. After incubation for 15 min at 14°C, the reaction was stopped with the addition of 10 mM Tris at pH 7.5. The cells were washed with PBS and observed with the phase-contrast microscope to ensure that cell clusters had not formed. The cells were lysed in cold triple detergent buffer (see section 1.2.1.1.3) by sonication on ice. The protein concentration was determined by the BCA protein assay and cell lysate was mixed with SDS sample buffer and analysed by Western blot.

2.2.3.10.Cell aggregation assay

HEK/EBNA suspension cells were transfected and incubated for 72 h following the protocol above. The cells were harvested by centrifugation at 250 x g and washed twice with Hank's buffered salt solution (HBSS) (Invitrogen) without calcium and magnesium. To obtain a single-cell suspension, cells were incubated for 15 min on a shaker at 37°C with cell dissociation solution (Sigma), and further dispersed by gentle pipetting. They were then suspended in HBSS without calcium and magnesium at a concentration of 1×10^6 cells/ml, placed in 12-well plates precoated with BSA, and rotated on a gyratory shaker for 20 min at 37 °C. Aggregation was stopped by the addition of 2% glutaraldehyde.

2.2.3.11.Isolating oligodendrocyte precursor cells from newborn mice

OPCs were isolated from newborn (P1-2) C57B6 mice (Charles River). Forebrains were extracted, washed with PBS, cut into pieces with a scalpel and incubated for 20 min at 37°C in 20 ml isolation medium (HBSS containing 0.9 mM NaHCO_3 , 100 U/ml penicillin, 100 µg/ml streptomycin and 25 mM HEPES buffer, pH 7.2) containing 0.025 % trypsin (Worthington) and 40 µg/ml DNase I type IV (Sigma). The reaction was stopped by addition of 1 ml of FBS. The volume was adjusted to 50 ml and the solution

centrifuged at $230 \times g$ for 5 min. The supernatant was filtered through a $150 \mu\text{m}$ nylon mesh and then further through a $65 \mu\text{m}$ nylon mesh. The cell suspension was placed on a Percoll gradient and centrifuged at $23\,500 \times g$ for 45 min (SS-34 rotor, Beckman). The gradient layer containing the OLs lies below the supernatant and myelin layer and above the red blood cell layer. This was collected and the cells were washed twice with isolation medium. The cell pellet was then resuspended in serum free Sato medium (DMEM containing GlutaMAX™ and sodium pyruvate (Invitrogen), $100 \mu\text{g/ml}$ BSA (fraction V) (Invitrogen), 40 nM Na-selenite (Sigma), 10 ng/ml D-biotin (Sigma), $5 \mu\text{g/ml}$ insulin (Sigma), $100 \mu\text{g/ml}$ apotransferrin (Sigma), 20 ng/ml forskolin (Sigma), $16 \mu\text{g/ml}$ putrescine (Sigma), 60 ng/ml progesterone (Sigma), $60 \mu\text{g/ml}$ Na-acetyl-cysteine (Sigma), 10 ng/mL PDGF-AA (R&D systems), and 10 ng/mL bFGF (Abcys S.A.) and $2\text{--}3 \times 10^6$ cells were plated per poly-D-lysine coated T25 flask. Cells were grown for 6 days before they were used for the agarose drop migration assay and the medium was changed daily.

2.2.3.12. Agarose drop migration assay

OPCs were isolated as described above and resuspended at 26×10^6 cells/ml or 52×10^6 cells/ml in modified Sato medium (DMEM (Invitrogen) supplemented with $5 \mu\text{g/ml}$ bovine insulin (Sigma), $50 \mu\text{g/ml}$ human transferrin (Sigma), $100 \mu\text{g/ml}$ BSA V (Sigma), 6.2 ng/ml progesterone (Sigma), $16 \mu\text{g/ml}$ putrescine (Sigma), 5 ng/ml sodium selenite (Sigma), 400 ng/ml T3 (Sigma), 400 ng/ml T4 (Sigma), 4 mM L-glutamine (Sigma), penicillin and streptomycin (Sigma)). Oli-neu cells were resuspended at 52×10^6 cells/ml in the same medium. Medium containing 40% FCS and 1.2% low melting point type VII agarose (Sigma) was added to the cell suspension in order to have a final concentration of 20×10^6 cells/ml or 40×10^6 cells/ml in Sato medium containing 10% FCS and 0.3 % agarose and the solution was kept at 37°C to prevent the agarose from solidifying. $1.5 \mu\text{l}$ drops of the cell suspension were applied to the center of the wells in 24-well tissue culture dishes and incubated for 15 min at 4°C to allow the agarose to solidify. The wells were either pre-coated with poly-D-lysine (BD biosciences) or $5 \mu\text{g/ml}$ poly-L-ornithine (Sigma) in H_2O . $400 \mu\text{l}$ of Sato medium with or without 5 ng/ml PDGF-AA was added to each well. For experiments with mice OPCs, bFGF (Abcys S.A.) was added at 5 ng/ml . For ECM substrates, the agarose drops were plated directly on the tissue-culture plastic, drops incubated for 15 min at 4°C and $50 \mu\text{l}$ $10 \mu\text{g/ml}$ laminin

(Sigma), fibronectin (Sigma) or Collagen I (Sigma) in Sato medium, or 50 μ l Matrigel (BD biosciences) was added around the drop. The cell cultures were incubated for 2 h at 37°C, after which 350 μ l Sato medium with or without 5 ng/ml PDGF-AA was added to each well. Test proteins were added to a final concentration of 0.0025, 0.025 or 0.25 μ M into the medium. PDGF-AA and test protein were added daily to the cell medium by replacing half of the medium with medium containing twice the concentration of the proteins. Cell migration was monitored daily using a phase contrast microscope (Nikon Diaphat 300) and documented with a camera (Nikon DXM 1200). We observed that the cells did not migrate in an uniform corona out from the drop and therefore recorded all sides in a total of 8 images.

The amount of migration was determined using an adapted program (created in-house) based on the ImageJ software. First, the drop was reconstituted by combining the images and the drop outline was defined. Then, the background was normalized amongst the merged images and finally the cells were identified due to the brightness of the round OPC cell bodies compared to the background. Both a threshold of brightness and the area of the bright spot were used to define a cell. Cell migration was determined by the median distance travelled by the cells from the drop circumference, hence discarding any discrepancies to the results caused by a minority of highly migrating cells. The number of cells migrating out from the drop was also determined. Within single experiments, each condition was tested in duplicate or triplicate. The results from three experiments are presented with standard deviation and statistical significance was determined using Bonferroni's multiple comparison test.

2.2.3.13. Proliferation in the agarose drop migration assay

Agarose drop migration assay was prepared in a poly-D-lysine coated 96 well plate, as described above with the only exception that 100 μ l of Sato medium with or without 2.5 μ M sGliaCAM or sCD147 was added instead of 400 μ l. Six wells were prepared per condition and agarose drops without cells were added as a control. [3H]-thymidine (TRK61, Amersham) was added at a final concentration of 10 nCi/ml to each well and the plate was incubated for 24 h at 37°C. At the end of the incubation, the plate was placed at -20°C in order to disrupt the cells. The plates were thawed, cells were lysed osmotically and the macromolecular DNA transferred onto a glass fiber filter mat (Wallac) using the FilterMate™ Universal harvester (Perkin Elmer). The filter mat was

dried for 4 min at 600 W in a microwave oven and sealed in a plastic bag containing 4 ml Betaplate Scint scintillation liquid (Wallac). Bound radioactivity was measured by scintillation counting in a MicroBeta® counter (Perkin Elmer).

2.2.3.14. Protein extraction from human brain samples

Snap frozen human brain tissue samples were received from the Brain Tumour Tissue Bank (Brain tumour foundation of Canada) and stored at -80°C until use. All the tumour tissues have been clinically characterised and graded. The supplier has further determined the quality of the sample by H&E (hematoxylin & Eosin) staining of a tissue section just adjacent to the one supplied. All the tissue samples that were used for this study are good quality (grading system includes good, moderate and poor), meaning a high ratio of tumour cells compared to hemorrhagic, necrotic and fibrous tissue contaminants. Furthermore, they are all from the tumour centre. The tissues were ground into powder by first tapping the tissue in a plastic bag gently with a hammer and then grinding it in a mortar. These steps were done in constant presence of liquid nitrogen to keep the temperature low. The tissue powder was then placed in a pre-cooled eppendorff tube and three times the powder volume of cold triple detergent buffer containing protease inhibitors was added to the tube. The tissues were homogenised using a polytron and the supernatant was collected after centrifugation at 89 000 x g (50 000 rpm in the TLA120.2 rotor (Beckman Instruments) for 20 min. The protein concentration was determined using the BCA protein assay kit and the samples analysed by Western blot as described above.

2.2.3.15. Culturing glioblastoma cell lines and 5-azacytidine treatment.

The human glioblastoma cell lines, A-172 and U-251, were grown in DMEM (Invitrogen) supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin (Sigma) and 10 % FCS (Sigma). The A-172 cells were plated at 50 000 cells per well in a 6-well-plate well. A 20 mM 5-azacytidine (Sigma) stock solution was prepared in 1:1 acetic acid to H₂O. The cells were incubated for 24 h with 1, 3 or 10 µM 5-azacytidine in the culture medium, then the cells were washed twice with drug-free medium and further incubated for various times in medium without 5-azacytidine. Control cells were incubated with equivalent volumes of 1:1 acetic acid to H₂O in culture medium compared to the 5-

azacytidine treated cells. Cells were lysed in triple detergent buffer on ice using a polytron, then centrifuged at 10 000 x *g* for 20 min and the supernatant was collected. Protein concentration was measure using a BCA protein assay kit (Pierce). Samples were analysed by SDS-PAGE and Western blot as previously described. U-251 cells were lysed in the same way for expression studies.

2.2.3.16.Quantitative PCR

See description above in published paper Favre-Kontula, L. et al, *Glia*, 2008.

2.3. Detection and identification of plasma proteins that bind GlialCAM using ProteinChip arrays, SELDI-TOF MS, and nano-LC MS/MS.

Favre-Kontula L, Sattonnet-Roche P, Magnenat E, Proudfoot AE, Boschert U, Xenarios I, Vilbois F, Antonsson B.

Proteomics. 2008 Jan;8(2):378-88.

Introduction

Characterising the molecular interactions of a protein, next to altering its expression levels or studying its subcellular localisation, has become an integral part of analysing its biological function. Proteins exert their function through dynamic, complex interactions, which make up protein complexes, that regulate normal and pathological cell states. Studying the proteome has gained a lot of interest in the postgenomic era. The knowledge that one gene can give rise to up to 50 different protein isoforms that are dynamically regulated within the cell demonstrates the challenge of proteomics (Cho, 2007). Functional proteomics assumes that protein interactions mean that the interacting proteins are involved in a common biological function. The ultimate goal of functional proteomics is to identify the molecular machinery of an entire cell by generating a map describing all molecular pathways, their functions, their reactions to external stimuli and their interconnectivity. Combined efforts from numerous research units will be required to combine the pieces of the functional proteomics puzzle. There are several methods to study functional proteomics, as shown in figure 46. These can roughly be divided into 1) affinity purification procedures from physiological fluids, 2) Pairwise testing of purified recombinant proteins, 3) Genetic readout systems, such as two-hybrid systems and 4) Computational prediction methods. A recent review by Köcher T. and Superti-Furga G. describes the different techniques in detail (Kocher and Superti-Furga, 2007).

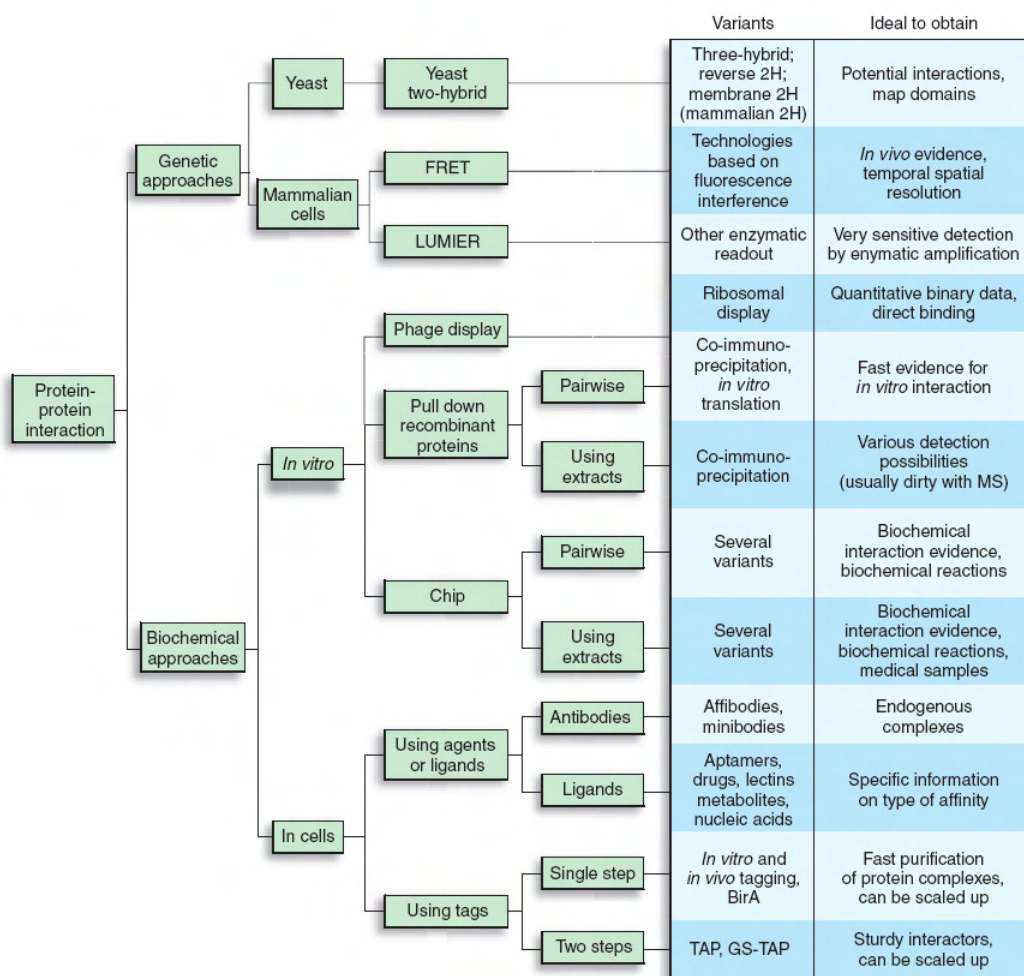


Figure 46. A decision tree showing the most common techniques used to study protein-protein (or protein-ligand) interactions. Several variations of the different methods are possible. 2H, two-hybrid. (Adapted from Köcher T. and Superti-Furga G., Nature Methods, 2007)

ProteinChips combined with SELDI-TOF MS is one method to capture protein-protein interactions, also shown in the figure above. ProteinChips and SELDI-TOF MS has been widely used for biomarker studies and to a lesser extent to develop process chromatography protocols and protein interactions studies, the latter mainly by using purified recombinant proteins and/or verifying already demonstrated interactions. There is to our knowledge only one report, prior to ours, demonstrating the use of ProteinChips and SELDI-TOF MS to capture and identify interacting proteins (Lehmann et al., 2005). Advantages of using ProteinChips combined with SELDI-TOF MS for interaction studies include 1) only a small amount of sample is needed, 2) it is less time-consuming than most other functional proteomics methods, 3) because of point 1 and 2, it is possible to

repeat experiments in order to confirm interactions and eliminate false positives, 4) Optimal interaction conditions can be identified by testing different buffer and incubation conditions, 5) peptides are easily detected, which is not the case when using gel electrophoresis, 6) protein complexes can be identified and not only pair-wise interactions as in 2-hybrid experiments, 7) the protein does not need to be tagged, 8) protein interactions are not masked by antibodies, which can be the case when using affinity purification.

LPS is a very potent activator of the innate immune system, as it is a major pathogen-associated molecular pattern (PAMP) for the recognition of invading gram-negative bacteria by the host. LPS consists of three parts, lipid A, a core of oligosaccharides and an O side chain, and is an important structural component of the outer membrane of gram-negative bacteria. The interaction of LPS with cells, including macrophages, granulocytes, dendritic cells and mast cells, leads to the synthesis and release of inflammatory mediators, such as tumor necrosis factor- α (TNF α), interleukin-1 (IL-1) and reactive oxygen species (ROS), which are essential for the activation and progression of the innate and subsequently adaptive immune response in order to combat bacterial infections. Host response to LPS is mediated mainly through Toll-like receptor 4 (TLR4) and the activation also requires lipopolysaccharide-binding protein (LBP), CD14 and MD-2 (Lu et al., 2008). Modest LPS induced activation of the immune response is important for eliminating invading micro-organisms, however a strong response can lead to a dysregulation of the innate immune response, which can have severe implications on the host, including multiple organ failure and death (Bosshart and Heinzelmann, 2007). Such strong responses can be caused by large numbers of bacteria, a high sensitivity of the host to LPS, or both. The clinical condition that evolves as a result of dysregulated host response to bacterial infection is called sepsis. Severe forms of sepsis occur in about two to three of 1000 hospital admissions, with mortality rates of 20% for patients with a severe form, 50% for patients with septic shock and 70% for patients with septic conditions associated with multiple organ failure (Brun-Buisson et al., 1996). There is a strong interest and commitment to develop pharmaceuticals that reduce mortality in septic patients.

The complement system is an integral part of innate immunity and plays a major role in controlling bacterial infections, especially those involving gram-negative bacteria. The complement system can be activated by at least three different enzymatic cascades:

classical, lectin and alternative (Carroll, 2004). These pathways differ in the recognition molecules that activate the pathway, but they all converge to generate C3 and C5 and the membrane attack complex (MAC). C3 and C5 are cleaved by serine proteases C3 and C5 convertase, respectively, rendering C3a/C3b and C5a/C5b. Nearly all the biological consequences of complement activation are dependent on the resulting cleavage products. Activated complement induces host defence against pathogens by opsonisation (flaging) through opsonins, including C3b, of microbes or toxic cell debris for subsequent elimination by MAC or phagocytic cells, by yielding anaphylatoxins C3a and C5a that promote chemotaxis and myeloid cell activation and by stimulating B cells to promote adaptive immunity (Gasque, 2004). MAC is composed of complement proteins C5b-8 that form a ringed structure with a membrane lytic activity, killing microbes. MAC does not discriminate between pathogen and host cells, so host cells control MAC activity through several mechanisms including soluble serum inhibitors and glycosyl phosphatidyl-inositol linked endothelial membrane proteins (Albrecht and Ward, 2005). The classical pathway is activated by IgG or IgM, the alternative pathway by microbial surface proteins or cellular injury and the lectin pathway by microbial surface proteins. LPS is a well known activator of the alternative and lectin complement pathways (Morrison and Kline, 1977). Even though, it has long been known that LPS is an activator of both the Toll-like receptor and the complement pathways and that they are both important for innate immunity, there are very few reports on whether and how they interconnect in host defense. A recent report demonstrates that the complement system is directly involved in regulating TLR4 induced cytokine production and release following LPS stimulation *in vivo* (Zhang et al., 2007). The model that is proposed by the authors for interconnected complement and TLR4 signalling following stimulation by LPS is shown in figure 47. The regulatory effect of complement mediated signalling is through the activated anaphylatoxins C3a and C5a, and their respective receptors. C3a and C5a stimulation leads to the amplification of the normal TLR4 dependent signal transduction and thereby to increased cytokine release. The target cells of C3a and C5a activation *in vivo* have not yet been fully determined, however, macrophages and/or endothelial cells may be among the responding cells. There is growing evidence that the complement system is involved the dysregulation of the innate immune response, leading to sepsis (Albrecht and Ward, 2005).

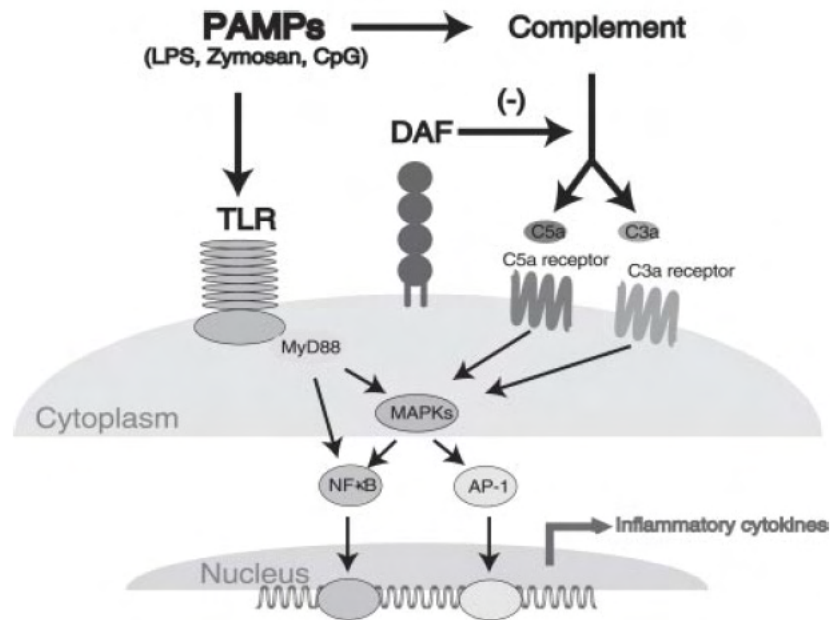


Figure 47. A schematic presentation of the proposed interaction between complement and the TLR pathway. PAMP activated complement regulates TLR signalling through the G-protein-coupled anaphylatoxin receptors, C3aR and C5aR, MAPKs and NF-κB, and likely other receptors. In the absence of complement regulatory protein DAF, complement activation and its effect on TLR signalling is amplified. Strong complement activators or pathological conditions such as sepsis may resemble complement activation in the absence of DAF. (From Zhang X. *et al.*, *Blood*, 2007)

Aims

Collaborators at the institute showed that sGliaCAM pre-administration reduces the TNFα cytokine response after LPS stimulation in mice. The aim of this work was to identify soluble proteins that interact with sGliaCAM in plasma from LPS stimulated mice, in order to understand how sGliaCAM exerts this effect. To search for proteins interacting with GliaCAM in plasma, a ProteinChips and SELDI-TOF MS based method was developed. Some of the reasons for choosing this method are indicated in the introduction above.

Summary

To identify how sGliaCAM pre-administration reduces the TNFα cytokine response after LPS stimulation in mice, we developed a novel strategy for the detection and

identification of protein binding partners. This encompassed using ProteinChips and SELDI-TOF MS to detect protein–protein interactions. Followed by a robust method for isolating and identifying proteins detected with the SELDI-TOF MS, using affinity beads, RP-HPLC, nano LC MS/MS and bioinformatic tools. It is one of the first reports of successfully using the SELDI-TOF MS method to identify protein-protein interactions from complex biological samples. We found several advantages in using this technique compared to other functional proteomics techniques. Firstly, sGlialCAM was covalently bound through its primary amino groups to the ProteinChip, which omitted the need for a specific antibody or to tag the protein. In 10–15% of cases the tag interferes with the function of the protein (Kocher and Superti-Furga, 2007). Furthermore, since sGlialCAM contains 11 primary amino groups and the protein is coupled randomly through the amino groups to the surface, different surfaces of the protein are likely to be exposed to the binding partners, although some amino groups might be more reactive than others. This could be an advantage to using a tagged protein and an anti-tag antibody, where the bait is immobilized in a single orientation. Using ProteinChips and SELDI-TOF MS, we were able to screen in a time and sample efficient way several different biological samples and incubation conditions for detection of sGlialCAM binding partners. With a standard immunoaffinity purification protocol we could have missed the binding partners, since they have approximately the same molecular weight as the antibody heavy chain, and even under non-reduced conditions traces of free antibody chains are present. Once we had identified specific peaks on sGlialCAM immobilized ProteinChips, the binding conditions were optimised in order to have a maximum amount of the interacting partners binding to sGlialCAM with a minimum amount of contaminating proteins. Again little sample and time was required for this step compared to more conventional proteomics methods. Once the optimal conditions had been determined, in order to have sufficient material for identification, sGlialCAM binding partners were isolated using affinity beads with immobilized sGlialCAM as on the ProteinChips. Proteins bound to the beads were eluted and analysed on a different type of ProteinChips that binds hydrophilic and charged residues, hence giving an overview of the protein composition in the solution. Bead eluates could therefore be directly compared to the results that had been obtained on ProteinChips with immobilized sGlialCAM. Since several proteins bound to sGlialCAM and low amounts of unspecific contaminating proteins were present, a further purification step was required. In proteomics two techniques are most frequently used, 1- and 2- dimensional gel electrophoresis and RP-HPLC. We opted for

the latter, because it does not require the digestion and peptide extraction step, which is necessary when using gel electrophoresis, and therefore protein yields are normally higher and there is less risk of introducing contaminations. In addition, RP-HPLC fractions containing the binding proteins can be identified according to their identified mass using the ProteinChips, which might not be identical to the apparent protein masses observed by electrophoresis. The purified binding proteins were digested in solution using trypsin and identified using nano LC MS/MS, through sequencing, peptide mapping and bioinformatic data base search. The method developed is not only applicable to protein interaction studies, but also for identifying biomarkers. Currently there are few reports on the identification of biomarkers detected by SELDI-TOF MS, even though it would significantly increase the confidence in the results and would allow the development of alternative assays that could be easier to use in routine clinical practice.

Using this novel method, we isolated and identified from the plasma of LPS stimulated mice, two proteins, mannose binding lectin C (MBL-C) and properdin, which interact with sGlialCAM. Traces of MASP-1 and MASP-2, which bind to MBL-C, were detected in the RP-HPLC fraction containing MBL-C, which might indicate that the complex is composed of several proteins, where MBL-C and properdin are the major contributors. These proteins function as activators of the inflammatory response following LPS stimuli and their sequestration would lead to decreased LPS response as was observed, suggesting that the interactions are relevant and specific. Furthermore, experiments in our laboratory show that GlialCAM interacts with the plant lectin concavalin A (Con A) (unpublished data) and therefore isolation of only two interacting proteins from a complex solution such as plasma where most proteins contain carbohydrate modifications indicated specific interactions.

Properdin is a soluble glycoprotein that positively regulates the alternative complement pathway by binding and stabilizing the inherently labile C3 and C5 convertase enzymes, C3bBb and C3bBbC3b, substantially increasing their half-lives (Fearon and Austen, 1975). The alternative complement pathway can be activated either independently or following activation of the classical or lectin pathway. In the latter case, low levels of C3 undergo “tick-over” to C3bBb. C3 cleavage by C3bBb to C3a and C3b can lead to the binding of C3b to an adjacent membrane surface and association with factor B to form C3bBb. Thus C3 cleavage can rapidly generate several membrane

bound C3bBb convertases. For this reason the alternative pathway is also referred to as the “amplification loop”. The 53 kD Properdin monomer is composed of an N-terminal domain and 6 thrombospondin type I repeat (TSR) domains, and contains a single N-glycosylation site. Under physiological conditions in plasma it exists as a mixture of oligomers, mainly dimers, trimers and tetramers in a ratio of approximately 1:2:1, with functional activity increasing with the size of the oligomer (Linton and Morgan, 1999). A recent study demonstrates that properdin can bind directly to microbial targets and provide a platform for C3bBb assembly, suggesting a major role for properdin in complement activation (Spitzer et al., 2007). Properdin can bind LPS directly and studies using properdin deficient mouse serum, demonstrate that properdin is indispensable for LPS induced complement activation (Kimura et al., 2008). Properdin seems to play a bigger role in independently activated compared to classical or lectin activated alternative complement pathway. sGliaCAM binding to properdin could prevent it from activating the complement pathway and thereby decrease complement enhanced TLR response to LPS, leading to decreased secretion of TNF α .

MBL is an evolutionary conserved host defense protein that following its binding to a variety of PAMPs activates the lectin complement pathway. MBL belongs to the collectin family. It has a bouquet like structure made up of several monomers composed of an N-terminal cysteine rich region, a collagen helix, a neck region and a C-terminal carbohydrate recognition domain (CRDs). In plasma, MBL oligomers associate with MASP (MBL-associated serine proteases) -1, -2, -3 and the non-enzymatic protein Map19. MBL specifically recognizes patterns of sugars such as mannose, N-acetylglucosamine and fucose on microbial surfaces. In addition to activating the lectin complement pathway MBL also works as an opsonin on microbial surfaces, thereby tagging the microbe for elimination by phagocytic cells. MBL activation lead to the formation of the classical pathway C3 convertase, C4b2a, and the activation is amplified through the alternative complement pathway. A recent paper suggests another pathway were MBL activates C3 and the alternative complement pathway in a C2 and MASP-1, -2 and -3 independent manner (Selander et al., 2006). It has been well established that MBL binds to the mannose rich portion of LPS through CRD and that the binding mediates complement activation (Brade and Brade, 1985; Swierzko et al., 2003). In human endothelial cell cultures, LPS stimulation enhances MBL binding to the cells (Oroszlan et al., 2007). Furthermore, following LPS stimulation the MBL/MASP complex or recombinant MBL increases the secretion of cytokines IL-8, IL-6 and MCP-1 through a

mechanism that required direct MBL binding to LPS. sGlialCAM could, in the same way as for properdin, prevent MBL activity by binding to the protein and thereby indirectly decrease LPS induced TNF α levels.

Taken together, sGlialCAM pre-administration reduces the TNF α cytokine response after LPS stimulation in mice, sGlialCAM binds MBL-C and properdin, both MBL-C and properdin function as activators of the complement pathway following LPS stimulation and a recent report shows that the complement system is directly involved in regulating TLR4 induced cytokine production and release following LPS stimulation (Zhang et al., 2007). Therefore, we suggest that sGlialCAM binding to MBL-C and properdin prevents their role as activators of the complement pathway following LPS stimuli and thereby TNF α levels are reduced compared to mice that have not been treated with sGlialCAM. However, further investigations are required to determine the role of sGlialCAM in complement activation. Whether soluble forms of GlialCAM exist naturally still remains to be determined, nevertheless the recombinant soluble form of the protein might be of therapeutic value.

RESEARCH ARTICLE

Detection and identification of plasma proteins that bind GlialCAM using ProteinChip™ Arrays, SELDI-TOF MS, and nano-LC MS/MS

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In order to fully understand biological processes it is essential to identify interactions in protein complexes. There are several techniques available to study this type of interactions, such as yeast two-hybrid screens, affinity chromatography, and coimmunoprecipitation. We propose a novel strategy to identify protein–protein interactions, comprised of first detecting the interactions using ProteinChips and SELDI-TOF MS, followed by the isolation of the interacting proteins through affinity beads and RP-HPLC and finally identifying the proteins using nano-LC MS/MS. The advantages of this new strategy are that the primary high-throughput screening of samples can be performed with small amounts of sample, no specific antibody is needed and the proteins represented on the SELDI-TOF MS spectra can be identified with high confidence. Furthermore, the method is faster and less labor-intensive than other current approaches. Using this novel method, we isolated and identified the interactions of two mouse plasma proteins, mannose binding lectin C and properdin, with GlialCAM, a type 1 transmembrane glycoprotein that belongs to the Ig superfamily.

Received: June 18, 2007
Revised: August 31, 2007
Accepted: October 12, 2007

Keywords:

GlialCAM / MS/MS / Plasma / Protein–protein interactions / SELDI-TOF

1 Introduction

A major challenge in biological research today is to understand the network of proteins composing functional biological complexes. The genomes of several species have been sequenced and characterization of the proteome is ongoing, leading to the next important step, namely studying the interactome. The interactome is defined by the complete set of protein–protein interactions in a cell or tissue. Protein–protein interactions are essential for the elucidation of pro-

tein function. These interactions are temporally and spatially regulated within a biological system and understanding the control mechanisms will give important insights into health and disease states.

Currently, there are several techniques available for studying protein–protein interactions. The most commonly used are yeast two-hybrid screens and different affinity purification techniques coupled to protein identification by 2-DE or multidimensional LC and MS. The available techniques have recently been described in comprehensive reviews [1, 2]. Affinity purification techniques for the isolation of protein complexes use either a direct capture with antibodies to the target protein or an indirect immobilization through the use of tagged proteins. If the protein complex is isolated through direct antibody capture, there is a prerequisite for an antibody with high affinity and specificity, which can be a limiting factor, in particular when working with novel proteins, due to the time required to raise the appropriate antibodies.

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Abbreviations: IDM beads, interaction discovery mapping beads; LPS, lipopolysaccharide; s.c., subcutaneously

Furthermore, the antibody binding site can be hidden in the protein complex, preventing its capture or steric hindrance can interfere with complex formation. With regard to tagged proteins, the tag may also mask potential protein binding sites. Moreover, protein–protein interaction assays are generally time-consuming and labor intensive.

SELDI-TOF MS has been developed over the last decade. This technology combines on-chip processing of biological samples with mass spectrometric analysis [3]. The most common use of the SELDI technology today is in biomarker research comparing serum from healthy and diseased individuals [4, 5]. The technology has also been used for the development of process chromatography protocols [6] and to follow protein processing *in vivo* [7]. Several recent reviews elaborate on the use of MS [1, 2] and more precisely SELDI-TOF MS [5, 8] for protein–protein interaction studies. However, there are only few reports demonstrating the entire process from the capture of interacting proteins from biological samples to their identification using SELDI-TOF MS technology [9]. The idea of using SELDI-TOF MS as a high-throughput analysis tool for protein interaction studies has been published by Howell *et al.* [10]. Generally, the SELDI-TOF MS technology has been used to verify known protein interactions [11, 12, 13]. Furthermore, it has been used to show protein–extracellular matrix [14] and protein–DNA [15] interactions. A method to identify proteins that bind to peptides has been described, however the identification of the putative interacting proteins has not been performed [16]. To our knowledge, there is only one report of the capture and identification of interacting proteins using the SELDI-TOF MS technology, demonstrating that S100A8 binds S100A10 [9].

We were interested in the function of a novel IgCAM protein that we named GlialCAM (Favre-Kontula, *et al.* submitted), the same protein sequence has recently also been published under the names hepaCAM [17, 18] and Zig-1 [19] (GeneID: 220296). GlialCAM is a single transmembrane protein, with two extracellular Ig domains and a proline rich C-terminal intracellular tail containing a putative SH3 domain-binding site and several potential phosphorylation sites [18]. In order to understand the function of this protein we decided to investigate potential binding partners using the extracellular domain of GlialCAM (GlialCAM-ECD). As an alternative to the conventional methods we have developed a SELDI-TOF MS and nano-LC MS/MS based method to screen for and identify protein–protein interactions. Our strategy comprised of a first part of primary screening of samples containing potential interacting proteins, followed by reconfirmation of positive interactions to minimize the effect of biological and technical variability and subsequent optimization of the interaction conditions between bait and prey proteins. All these steps were performed on ProteinChips and analyzed on SELDI-TOF MS. The second part was the isolation of the prey proteins using interaction discovery mapping beads (IDM affinity beads) and RP-HPLC. At each step of the isolation, the samples containing the interacting

proteins were verified on the ProteinChips using SELDI-TOF MS. Finally, the identification of the interacting proteins was performed by tryptic digestion, nano-LC MS/MS sequencing, and bioinformatic database searches.

Lipopolysaccharide (LPS), the active component of endotoxin from Gram-negative bacteria, can cause an excessive activation of the innate immune system with subsequent uncontrollable cytokine release. This can lead to cardiovascular collapse and hemodynamic instability and can provoke a fatal sepsis syndrome in humans. Sepsis is a significant cause of mortality in critically ill patients [20]. A lot of effort has been undertaken to find therapies against LPS-induced sepsis [21, 22, 23]. Proteins containing Ig domains play a central function in immune responses, since approximately a third of the cell-surface receptors expressed on leukocytes belong to the Ig superfamily (IgSF) [24]. We observed that the extracellular domain of GlialCAM reduced LPS-induced increase in circulating levels of TNF α when administered to mice, and therefore we used plasma from these mice as the source to search for proteins that interact with GlialCAM. Plasma is one of the more challenging samples to work with in proteomics research due to its complexity and huge dynamic range, *e.g.*, hormones and cytokines at pg/mL levels or less and albumin, transferrin, and IgG at mg/mL levels. It has been suggested that mouse plasma contains most, if not all, murine proteins [25]. Proteins are introduced to plasma from all tissues either through regulated secretion or from cells undergoing cell death and lysis. Most proteins are likely to exist with different PTMs, which further increases the complexity. Frequently, the low abundant proteins are of most interest in proteomics research.

We report here the use of ProteinChips and SELDI-TOF MS to detect protein–protein interactions. Further, we propose a robust method for isolating and identifying proteins detected with the SELDI-TOF MS, a strategy that can also be applied to identify biomarkers. Using this novel method, we isolated and identified from the plasma of LPS stimulated mice, two proteins, mannose binding lectin C (MBL-C) and properdin, which interact with GlialCAM.

2 Materials and methods

2.1 Reagents

Recombinant human interleukin 18 binding protein isoform a (IL-18BP α) was obtained from Serono manufacturing facilities. Recombinant human interleukin 18 (IL-18) was expressed in *Escherichia Coli* and purified from the inclusion bodies. In summary, the inclusion bodies were solubilized and the protein purified under denaturing conditions by LC on Sephacryl S-200 (GE Healthcare, USA) and Q-Sepharose (GE Healthcare) columns. After renaturation through dialysis the renatured protein was finally purified by gel filtration on Superdex 75 (GE Healthcare). His tagged GlialCAM

was expressed in HEK cells and purified on Ni metal affinity chromatography followed by gel filtration on Superdex 200 (GE Healthcare).

2.2 Analysis of the integrity and purity of the bait protein on the NP20 ProteinChip

The bait protein (1.25 µg in 5 µL) was applied and air-dried onto the normal phase NP20 ProteinChip Arrays (BioRad Laboratories, USA). Proteins bind through hydrophilic and charged residues to the silicon oxide groups of the NP20 ProteinChip. The spot was washed five times with 5 µL H₂O and air-dried. A saturated sinapinic acid solution in 50% ACN, and 0.5% TFA in water was applied to each spot on the ProteinChips and mass analysis was performed by SELDI-TOF MS, using the ProteinChip™ Biology System II and Ciphergen software version 3.0. Spectra were generated using laser intensity 180, sensitivity 9, and mass focus between 10 and 70 kDa.

2.3 Murine model of LPS induced immune response

GlialCAM (1 mg/kg) or saline was injected s.c. into female C3H mice. Two hours later 0.3 mg/kg LPS or saline was administered s.c. and after 1.5 h TNFα levels were determined by ELISA. For protein–protein binding experiments, the mice were injected s.c. with 0.3 mg/kg LPS or saline and the blood collected intracardially after 3 h.

2.4 Protein–protein interaction studies using ProteinChips and SELDI-TOF MS

The bait or control protein (1.25 µg in 5 µL) was immobilized to each spot on RS100 ProteinChip Arrays (BioRad Laboratories) through incubation for 2 h at room temperature in a humidified chamber. Primary amino groups of the proteins bind covalently with the preactivated carbonyl diimidazole functional groups on the RS100 ProteinChips. All subsequent incubations were performed on a shaker at room temperature. Unreacted amine groups were blocked with 0.5 M ethanolamine/PBS for 1 h. The ProteinChips were washed three times for 5 min with PBS containing 0.1% Triton X-100. Plasma samples were diluted 1:1 in PBS, containing 0.1% Triton X-100 and EDTA-free protease inhibitor cocktail (Roche Diagnostics), and 50 µL were added to GlialCAM and IL-18 immobilized spots, respectively. The ProteinChips were then washed twice for 5 min with PBS containing 0.1% Triton X-100 and once for 3 min with Tris-HCl buffer, pH 9.0, containing 1 M Urea, 2% CHAPS, and 0.5 M NaCl. The ProteinChips were rinsed with 5 mM HEPES pH 7.2 for 15 s and air-dried. A saturated sinapinic acid solution in 50% ACN, and 0.5% TFA in water was applied to each spot on the ProteinChips and mass analysis was performed by SELDI-TOF MS, using the ProteinChip Biology System II and Ciphergen software version 3.0 (Ciphergen Biosystems, Fremont, CA, USA). Spectra were generated

using laser intensity 180 or 200, sensitivity 9 or 10, and mass focus between 10–40 and 20–160 kDa, to identify lower and higher molecular weight binding partners, respectively.

2.5 Data analysis to detect proteins binding to GlialCAM

The spectra were calibrated using the all-in-one protein standard (Hirudin 7034 Da, Cytochrome *c* (Bovine) 12 230 Da, myoglobin (Equine) 16 951 Da, carbonic anhydrase (Bovine) 29 023 Da, enolase (*Saccharomyces cerevisiae*) 46 671 Da, albumin (Bovine) 66 433 Da, IgG (Bovine) 147 300 Da) from Ciphergen Biosystems, following the manufacturers' instructions. Peaks in the spectra obtained with Ciphergen's technology platform and ProteinChip software were annotated using the average neutral mass of the peptide or protein followed by their protonated charge state. Normalization of signal intensity of all spectra was carried out using the TIC. Protein peaks present on the GlialCAM spots but absent on the control spots were detected using the Ciphergen's Biomarker Wizard software.

2.6 Affinity purification using IDM affinity beads

IDM affinity beads (Ciphergen Biosystems) were used for the isolation of the interacting proteins for subsequent identification. A similar protocol was applied with the beads as that used for the RS100 ProteinChip. One milligram of bait protein in 50 mM sodium acetate buffer, pH 5.0, in a total volume of 600 µL was incubated with 200 µL beads overnight at room temperature. The beads were washed twice for 5 min with 1 mL 50 mM sodium acetate buffer, pH 5.0, and then blocked with 1 mL 0.5 M Tris-HCl, pH 9, containing 0.1% Triton X-100, for 2 h. They were washed three times for 10 min with 1 mL PBS containing 0.1% Triton X-100 and twice for 10 min in 1 mL PBS. Mouse plasma was diluted 1:1 in PBS containing 0.1% Triton X-100 and EDTA-free protease inhibitor cocktail (Roche Diagnostics) and 1200 µL were added to GlialCAM and IL-18 immobilized beads, respectively. The beads were incubated with the samples overnight at 4°C on a roller. The beads were then washed twice with 1 mL 50 mM Tris-HCl, pH 7.5, containing 0.2 M Urea, 0.1 % CHAPS, and 0.2 M NaCl, and twice with 1 mL PBS for 5 min. Interacting proteins were eluted with 200 µL water containing 50% ACN and 0.3% TFA. The eluates were analyzed on NP20 ProteinChips. Three microliters of eluate were added to the respective spots on a NP20 ProteinChip and incubated for 10 min at room temperature. The spots were washed twice with 5 µL H₂O and 0.8 µL of saturated sinapinic acid solution in 50 % ACN and 0.5 % TFA in H₂O were added to the spots and the ProteinChips were then air dried. Spectra were generated using a laser intensity of 180 or 200, and a sensitivity of 9 or 10 and mass focus between 10–40 and 20–160 kDa, to identify lower and higher molecular weight proteins, as above. The elu-

ates were also analyzed by SDS-PAGE followed by staining with silver nitrate. The remaining eluate was lyophilized for RP-HPLC fractionation.

2.7 RP-HPLC for isolation of the individual proteins

The eluate was fractionated on a 4.6 mm × 50 mm RP C4 (butyl) column (VYDAC 214TP™ Series, Grace Vydac), with a linear gradient of 0–90% ACN in 0.1% TFA at a flow rate of 0.2 mL/min. Two hundred microliters fractions were collected. An aliquot of each fraction was analyzed on a NP20 ProteinChip.

2.8 Tryptic digestion of proteins for MS identification

The RP-HPLC fractions containing the individual interacting proteins were pooled, lyophilized, and resuspended in 50 µL 0.2 % (w/v) Rapigest™ SF (Waters, USA). The tryptic digest was performed following the protocol provided by the manufacturer, in brief the samples were reduced with DTT at a final concentration of 5 mM, alkylated with iodoacetamide at a final concentration of 15 mM, incubated for 60 min at 37°C with 1:100 (w/w) trypsin to sample and the reaction was stopped by addition of HCl to a final concentration of 40 mM.

2.9 Nano-LC MS/MS analysis and protein sequencing

The mass spectrometric analysis was performed using a Q-Star-XL quadrupole/TOF instrument (MDS/Sciex, Canada) and the acquisition software Analyst QS, version SP7. The interface was connected to an Ultimate HPLC system (LC-Packings, USA), equipped with a capillary C-18 RP column (75 µm inner diameter, 150 mm length) packed with Pep-Map C18, 3 µm, 100 Å. Five microliters peptide digest were

injected onto the column using a Famos autosampler (LC-Packings) and separated with a 60 min 5–60% ACN gradient in 0.1% formic acid at a flow rate of 200 nL/min. The column was connected to a nanoemitter (New Objective, USA) and the eluent sprayed towards the orifice of the mass spectrometer using a potential of 2400 V. The mass spectrometer was operated in data dependent acquisition mode. A second survey scan was performed in the MS mode followed by four MS/MS scans when $[M + 2H]^{2+}$ or $[M + 3H]^{3+}$ precursor ions are detected above a signal of 20 counts. Tandem mass spectra were obtained using nitrogen as CID gas at collision energies that were set automatically depending on the mass and the charge of the precursor ion. The sequences of the peptides were determined after transfer of the MS/MS data to the MASCOT program and searches were performed in protein databases. The search parameters for MASCOT were the following. Type of search: MS/MS ion search; enzyme/trypsin; fixed modifications: carbamidomethyl (c); mass values: monoisotopic; protein mass: unrestricted; peptide mass tolerance: ± 0.8 Da; fragment mass tolerance: ± 0.15 Da; maximum missed cleavages: 1; and instrument type: ESI-QUAD-TOF. Database searched: MSDB (November 2003) containing 1 268 262 sequences, with no restriction on species.

3 Results

3.1 Validation of the RS100 ProteinChip method using purified recombinant proteins that are known to interact

The strategy used in this study to detect and identify protein–protein interactions is presented in Fig. 1. First, we tested whether in our hands and with our protocol we could detect

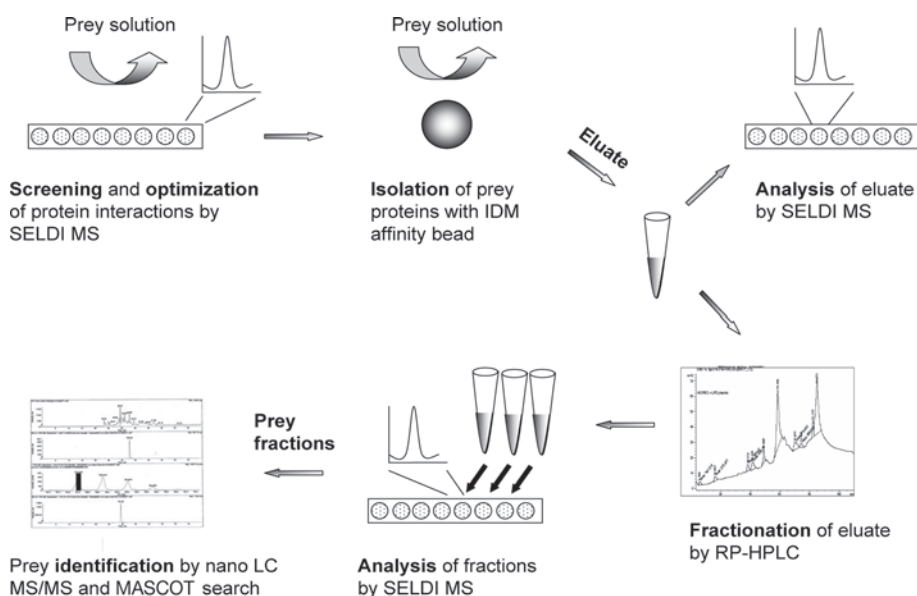


Figure 1. Schematic presentation of the novel strategy to identify protein–protein interactions with ProteinChips, SELDI-TOF MS, and nano-LC MS/MS. Interacting proteins are first detected using ProteinChips and SELDI-TOF MS, then isolated with IDM affinity beads and RP-HPLC and finally identified using nano-LC MS/MS and database searches.

protein–protein interactions using RS100 ProteinChips. For this verification we used IL-18 and IL-18BP_a, two proteins known to interact and form a complex.

3.1.1 Testing the integrity and purity of the bait and prey proteins using the NP20 ProteinChip

First, the integrity and purity of the IL-18 and IL-18BP_a protein samples were verified on the NP20 ProteinChip. As shown in Fig. 2a, IL-18 and IL-18BP_a were intact with a m/z of 18 398 (MW_{theo} 18 347.9 Da) and 30 187 (MW_{theo} 17 872.3 Da), respectively. The difference between the observed and theoretical mass of IL18-BP_a is due to glycosylation of the protein [26]. No contaminating proteins were detected in the solutions. Testing the integrity of the purified proteins after storage periods and sometimes even repeated freezing and thawing is important at the beginning of an interaction experiment, but is often not performed due to time constraint and/or limited amounts of sample. With the NP20 ProteinChip and SELDI-TOF MS only a few microgram of sample are needed and it takes only about 30 min.

3.1.2 IL-18BP_a binding to IL-18 immobilized on the RS100 ProteinChip

IL-18 was covalently bound through its primary amino groups to the preactivated carbonyl diimidazole functional

groups on the RS100 ProteinChip. After washing and blocking nonreacted groups on the ProteinChip surface with ethanolamine, the ProteinChip spots were incubated with IL-18BP_a or PBS (control). As shown in Fig. 2b, only the ProteinChip spot containing immobilized IL-18 bound IL-18BP_a (lower spectra). No IL-18BP_a binding could be detected on the uncoated spot (upper spectra) and the spot with immobilized IL-18 and incubated with PBS showed no peak corresponding to IL-18BP_a (middle spectra). However, a peak corresponding to IL-18 was detected which could be attributed to homodimeric IL-18 that was not dissociated during the washing steps. Taken together, this demonstrates that protein–protein interactions can be detected, with our protocol and in our hands, after direct coupling of the purified bait protein to the RS100 ProteinChip and subsequent incubation with the purified prey protein.

3.2 Identification of plasma proteins interacting with GlialCAM immobilized to RS100 ProteinChips

As shown in the proof of concept experiment described above, proteins interacting with a protein immobilized on a RS100 ProteinChip can be detected. We therefore decided to test the method for a protein of unknown function, GlialCAM, on more complex samples.

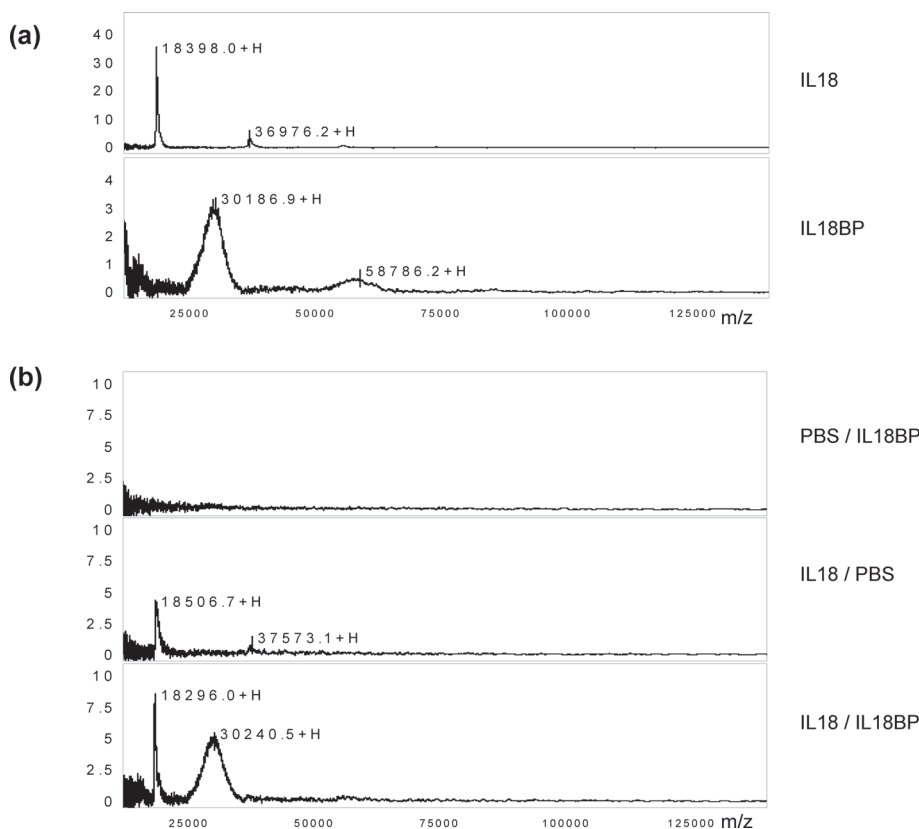


Figure 2. Detection of IL-18 interaction with IL-18BP_a on the RS100 ProteinChip. (a) The MS spectra of purified IL-18 (upper spectra) and IL-18BP_a (lower spectra) were determined using the NP20 ProteinChip. (b) RS100 ProteinChips containing immobilized IL-18, IL-18BP_a, or no immobilized protein (PBS) were incubated with either purified IL-18BP_a or PBS and subsequently analyzed on SELDI-TOF MS. The mass peak at 30 240 m/z corresponding to IL-18BP_a was present only in the spectra from the spot with immobilized IL-18 and after incubation with IL-18BP_a (lower spectra). No mass peak corresponding to IL-18BP_a was detected on the uncoated spot (upper spectra) nor on the spot immobilized with IL-18 without incubation with IL-18BP_a (middle spectra). The peak at 18 000 m/z that is seen in the middle and lower spectra, corresponds to the IL-18 monomer.

3.2.1 LPS induced inflammation *in vivo*

The extracellular domain of GlialCAM reduces the generation of $\text{TNF}\alpha$ induced by LPS in a murine model. As shown in Fig. 3, when GlialCAM was administered prior to the administration of LPS, the $\text{TNF}\alpha$ levels were reduced compared to the control animals. GlialCAM administration alone did not influence the level of $\text{TNF}\alpha$. This suggests that the extracellular domain of GlialCAM exerts an inhibitory function on the induction of $\text{TNF}\alpha$, presumably through interactions with other proteins.

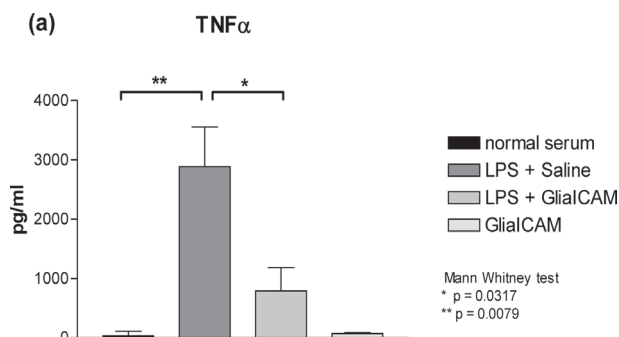


Figure 3. GlialCAM-ECD reduced LPS induced $\text{TNF}\alpha$ levels in mice. Female C3H mice were injected s.c. with the extracellular domain of GlialCAM, or saline, 2 h prior to the s.c. administration of LPS. Plasma was collected after 1.5 h for ELISA analysis of $\text{TNF}\alpha$ levels. The data represent the mean $\pm$ SD for four animals per condition.

3.2.2 Testing the integrity and purity of the bait proteins using the NP20 ProteinChip

The integrity and purity of the purified bait protein, recombinant GlialCAM-ECD, was analyzed on NP20 ProteinChips. The mass spectra presented in Fig. 4a shows that the protein has the expected molecular mass of approximately 32 800 Da and no contaminating proteins could be detected in the sample. The theoretical molecular weight of the recombinant GlialCAM-ECD is 23 879 Da, but due to PTMs in form of glycosylation, the observed molecular weight by MALDI-TOF MS is approximately 32 800 Da.

3.2.3 Detection of specific plasma proteins that bind to GlialCAM immobilized on RS100 ProteinChips

Plasma was collected from mice 3 h after LPS or saline administration. The plasma from LPS and saline treated animals was incubated on RS100 ProteinChip spots containing immobilized GlialCAM or IL-18. As shown in Fig. 4b, two specific protein peaks at 51 and 56 kDa, were detected on ProteinChip spots carrying immobilized GlialCAM but were absent on the control spots. The intensity of these protein peaks on spots incubated with plasma from LPS treated mice were approximately twice those observed on spots incubated with plasma from saline treated mice. The whole experiment was repeated three times with separate biological samples and for each experiment the samples were analyzed in

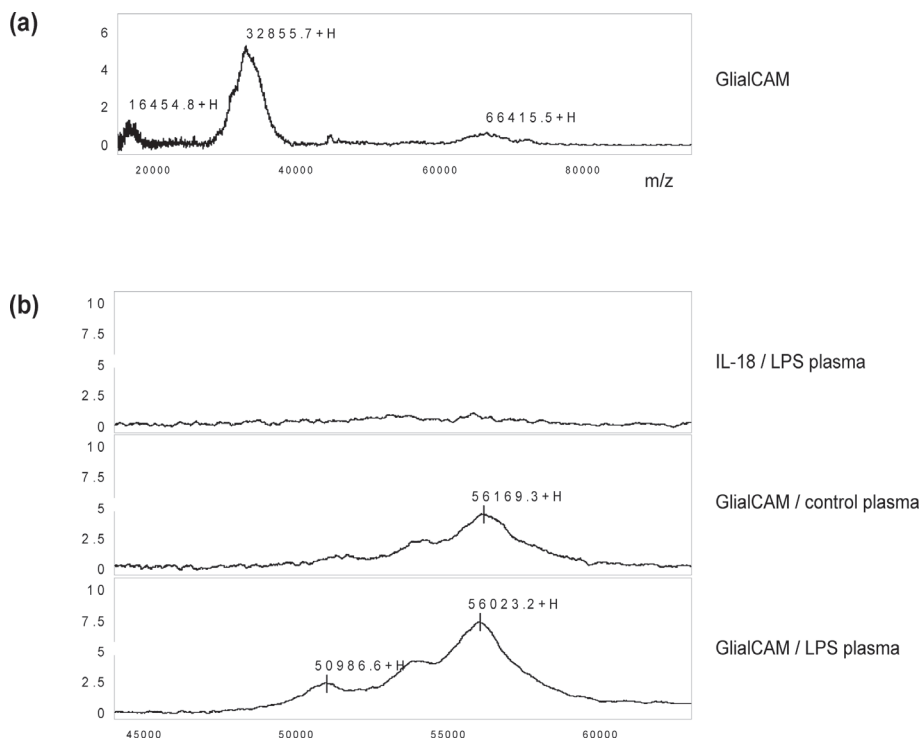


Figure 4. Detection of specific plasma proteins binding to GlialCAM immobilized to the RS100 ProteinChip. (a) Analysis of the purified GlialCAM-ECD sample on the NP20 ProteinChip. (b) RS100 ProteinChip spots containing immobilized IL-18 (upper spectra) or GlialCAM-ECD (two lower spectra), were incubated with plasma from LPS or saline treated mice as indicated in the figure. The spectra were generated with a laser intensity of 200, a sensitivity of 10, with a mass focus of 20–160 kDa m/z , and normalized to the TIC.

quadruplicates and the two peaks were reproducibly observed. Different incubation times (Fig. 5a) and washing conditions (data not shown) were tested in order to have maximal binding of the potential interacting partners to GlialCAM for subsequent isolation and identification of the proteins. As shown in Fig. 5a, incubation for 6 h resulted in higher peak intensities compared to 2 h incubation. Washing with 50 mM Tris, pH 7.5, containing 0.2 M Urea, 0.1 % CHAPS, and 0.2 M NaCl gave more intense peaks compared to those obtained after washing with 0.1% Triton X-100 in PBS (data not shown).

3.3 Scale-up for isolation of the interacting proteins on IDM affinity beads for protein identification

The optimal conditions that had been established for the interactions on RS100 ProteinChips, were used for the scale-up isolation of GlialCAM interacting proteins on IDM affinity beads. The beads have the same carbonyl diimidazole chemistry as the ProteinChips, but allow for isolation of larger amounts of interacting proteins as required for their subsequent identification. GlialCAM and IL-18 were coupled directly to the beads and after washing and blocking of unreacted groups with ethanolamine, the beads were incubated with plasma collected from LPS and saline (results not shown) treated mice. Proteins bound to the beads were eluted and analyzed on NP20 ProteinChips. The same peaks at 51 000 *m/z* and 56 000 *m/z* as seen on RS100 ProteinChips were found to bind to GlialCAM immobilized to the beads (Fig. 5b). At

51 000 *m/z* no peak was detected in the eluate from the IL-18 coupled beads, however, at 56 000 *m/z* there was a trace peak also in the spectra of the eluate from the IL-18 coupled beads. Also, a weak peak at approximately 53 000 *m/z* could be detected in the eluate of both the GlialCAM and IL-18 immobilized beads. Since this peak was present in both GlialCAM and control conditions it was not further analyzed.

3.4 Isolation of the interacting proteins using RP-HPLC

The interacting proteins eluted from the beads with immobilized GlialCAM were fractionated by RP-HPLC on a C4 column (Fig. 6a). The fractions eluted from the column were analyzed on NP20 ProteinChips. As seen in Fig. 6a and b the 56 and 51 kDa proteins eluted in fractions 57–60 and 83–85, respectively. The fractions containing each protein were pooled, respectively, and further analyzed by SDS-PAGE under reducing conditions and the proteins visualized by silver nitrate staining (Fig. 6c). The protein in the fractions 83–85 had an apparent molecular weight of approximately 30 kDa on SDS-PAGE whereas in SELDI-TOF MS analysis it had a molecular weight of approximately 51 kDa. The discrepancy could be due to the formation of disulfide bridged dimers that are dissociated under the reducing conditions used for SDS-PAGE analysis. The protein in the second pool containing the 56 kDa protein migrated at an apparent molecular weight of approximately 60 kDa, close to the mass determined by SELDI-TOF MS.

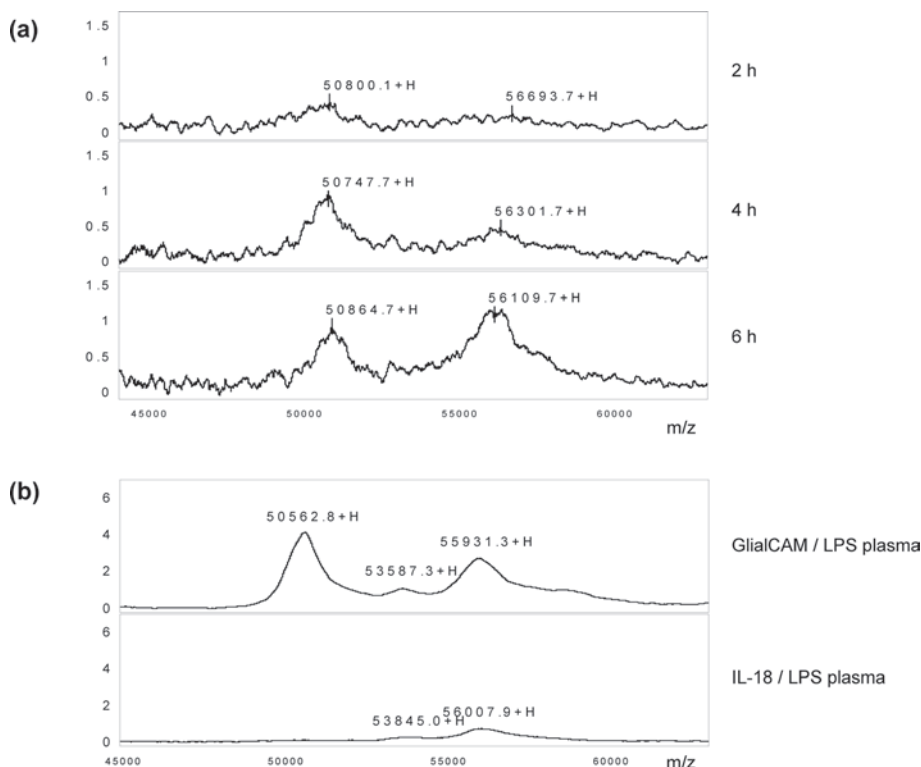


Figure 5. Determination of optimal conditions for binding of the 51 and 56 kDa protein to GlialCAM and scale-up isolation on IDM beads. (a) Plasma from LPS treated mice was incubated for different time periods on the RS100 ProteinChip spots with immobilized GlialCAM to find the optimal conditions for binding of the interacting proteins. (b) GlialCAM and IL-18, as a control protein, were immobilized on IDM beads and the beads were subsequently incubated with mouse plasma. Proteins bound to the beads were eluted and analyzed by SELDI-TOF MS. The two proteins at 51 and 56 kDa, which had been detected with the RS100 ProteinChip, were also detected in the spectra of the eluate from GlialCAM. No protein peak at 51 kDa was detected in the control eluate, however, a minor peak at 56 kDa was detected. In both eluates a small peak at 53 kDa could be detected.

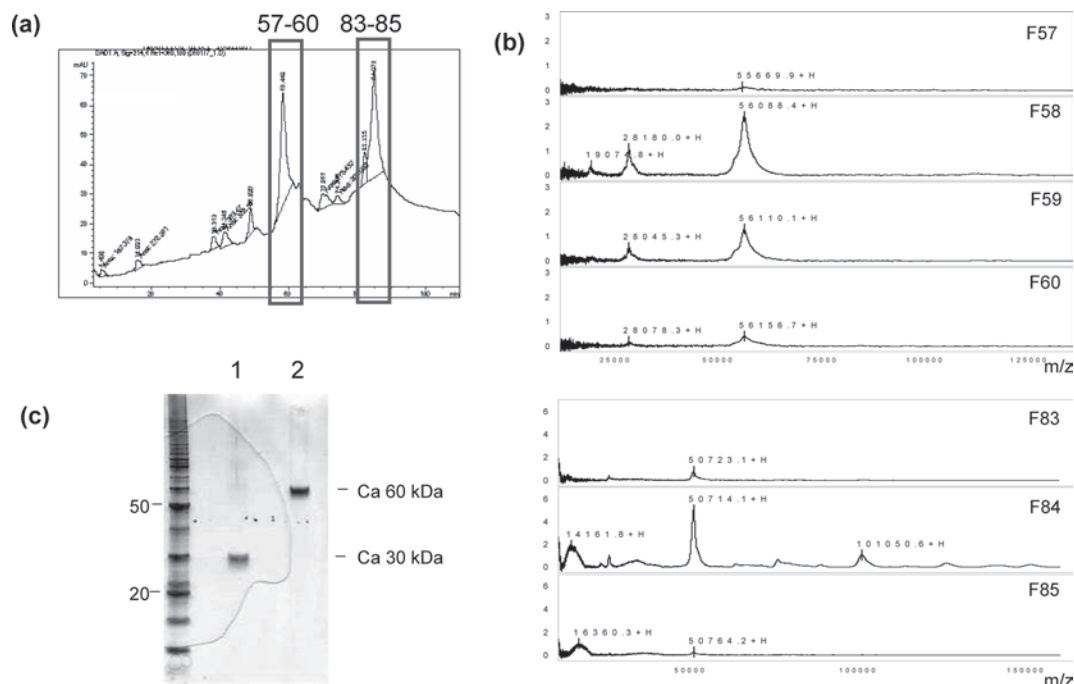


Figure 6. RP-HPLC fractionation of the bead eluate for isolation of the individual interacting proteins. (a) Eluate from IDM beads with immobilized GlialCAM was fractionated on a C4 RP column using a 0–90% ACN gradient in 0.1% TFA. (b) The fractions eluted from the RP-HPLC column were analyzed on the NP20 ProteinChip. The 56 kDa protein eluted in fractions 57–60 and the 51 kDa protein in fractions 83–85. (c) The fractions containing the 51 and 56 kDa proteins were pooled, respectively, and analyzed by SDS-PAGE electrophoresis and silver nitrate staining. Lane 1; pool F83–85, Lane 2; pool F57–60. Benchmark protein ladder (Invitrogen) was used as molecular weight marker.

3.5 Identification of the proteins by nano-LC MS/MS and database searches

In order to identify the isolated proteins, digestion with trypsin followed by sequencing of the tryptic peptides by nano-LC MS/MS was performed. As shown in Fig. 7a, with four sequenced peptides and 28% sequence coverage, MASCOT searches identified the 51 000 *m/z* protein as MBL-C. The 56 000 *m/z* protein was identified as properdin with five sequenced peptides and 17% sequence coverage (Fig. 7b). Both proteins are implicated in the complement pathway. The observed molecular weights correspond to the masses expected for these two proteins. MBL-C has a theoretical molecular weight of 24 kDa but is known to form higher oligomers through disulfide bonds and properdin has a calculated molecular weight of 50 kDa and is known to be glycosylated.

4 Discussion

We have used the SELDI-TOF MS ProteinChip technology to capture mouse plasma proteins that interact with the extracellular domain of GlialCAM. This is one of the first reports that demonstrates the successful use of SELDI-TOF MS to identify novel protein–protein interactions from complex

biological samples. We found several advantages of using SELDI-TOF MS compared to more conventional methods. A main advantage is that a large number of samples can be tested with only a small amount of sample required for each test, a crucial point when working with tissue biopsies and bio-fluids. The combination of a fast process and low sample consumption permits replicate analysis of each sample, which can compensate for sample and technical variability frequently associated with interaction studies. In addition, the ProteinChip technology allows rapid screening of a large number of parameters, such as incubation buffer, incubation time, and wash conditions. The bait protein can be immobilized either through direct covalent coupling to the ProteinChip surface or through an immobilized antibody. Lehmann *et al.* [9] in their study of protein interactions used the antibody approach for the immobilization. An advantage with the method we propose where the bait protein is bound through its amino groups directly to the ProteinChip surface, is that specific antibodies, which take considerable time to produce and characterize, are not required. GlialCAM has 11 primary amino groups distributed throughout the primary amino acid sequence. Since charged amino acids are frequently present on the surface of the protein it is likely that the protein is coupled through different amino groups to the reactive groups on the ProteinChip surface, giving a random orientation of the bait molecule, exposing different

(a)

1 MSIFTSFLL CVVTVVYAET LTEGVQNSCP VVTCSSPGLN GFPGKDGRDG
 51 AKGEKGEPGQ GLRGLQGPPG KVGPTGPPGN PGLKGAVGPK GDRGDRAEFD
 101 TSEIDSEIAA LRSELRALRN WVLFSLSEK GKKYFVSSVK KMSLDRVKAL
 151 CSEFQGSVAT PRNAEENSAI QKVAKDIAYL GITDVRVEGS FEDLTGNNRV
 201 YTNWNDGEPN NTGDGEDCVV ILGNGKWNV PCSDSFLAIC EFSD

Start - End	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Sequence	Ion score
97 - 112	883.90	1765.79	1765.84	-0.05	0	AEFDTSEIDSEIAALR	73
120 - 129	611.80	1221.59	1221.64	-0.05	0	NWVLFSLSEK	38
147 - 162	583.90	1748.68	1748.89	-0.21	1	VKALCSEFQGSVATPR	47
149 - 162	583.90	1748.68	1748.89	-0.21	0	ALCSEFQGSVATPR	89
173 - 186	511.90	1532.68	1532.86	-0.18	1	VAKDIAYLGITDVR	69
176 - 186	618.30	1234.59	1234.66	-0.07	0	DIAYLGITDVR	56
187 - 198	662.20	1322.39	1322.61	-0.22	0	VEGSFEDLTGNNR	80
187 - 200	789.80	1577.59	1577.78	-0.19	1	VEGSFEDLTGNNRV	76

(b)

1 MPAEMQAPQW LLLLLVILPA TGSDPVLCT QYEESGRCK GLLGRDIRVE
 51 DCCLNAAYAF QEHDGGLCQA CRSPQWSAWS LWGPCSVTCS EGSQLRHRR
 101 VGRGGQCSSEN VAPGTLEWQL QACEDQCCP EMGGWSEWGP WGPCSVTCSK
 151 GTQIRQRVCD NPAPKCGGHC PGEAQSSQAC DTQKTCPTHG AWASWGPWSP
 201 CSGSCLGGAQ EPKETRSRSC SAPAPSHQPP GKPCSGPAYE HKACSGLP
 251 PVAGGWGPWS PLSPCSVTCT LGQTLQRTD DHPAPRHGGP FCAGDATR
 301 MCNKAIVPCPV NGWEAWGKW SDCSRLRMSI NCEGTPGQQR RSRSCGGRKF
 351 NGKPCAGKLQ DIRHCYNIHN CIMKGSWSQW STWSLCTPPC SPNATRVRRQ
 401 LCTPLLPKYP PTVMVEGQG EKNVTFWGTG RPLCEALQGQ KLVVEEKRSC
 451 LHVPVCKDPE EKKP

Start - End	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Sequence	Ion score
219 - 242	849.70	2546.08	2546.14	-0.06	0	SCSAPAPSHQPPGKPCSGPAYEHK	27
287 - 298	623.20	1244.39	1244.54	-0.15	0	HGGPFCAAGDTR	33
326 - 341	611.90	1832.68	1832.86	-0.18	1	LRMSINCEGTPGQQR	57
301 - 314	782.80	1563.59	1563.68	-0.09	0	MSINCEGTPGQQR	55
399 - 408	613.30	1224.59	1224.70	-0.12	1	QRLCTPLLPK	25
449 - 462	566.50	1696.48	1696.79	-0.31	1	SCLHVPVCKDPEEK	44

Figure 7. Identification of the interacting proteins as mouse mannose-binding lectin C (a) and properdin (b) by nano-LC MS/MS. Matched peptides are underlined in the sequence and details for their identification by MASCOT are below. The signal sequences are shown in italics.

surfaces for interacting partners. This could be an advantage compared to the use of tagged bait proteins and an anti-tag antibody where the protein molecule will be immobilized in a specific orientation. For small proteins or peptides with few primary amino groups the direct coupling might not be the best choice, however, this can be overcome by using fusion proteins [16]. The use of immuno- or direct-immobilization should be assessed depending on the properties of the bait protein and the availability of specific antibodies. It should also be kept in mind that covalent attachment can sometimes lead to loss of binding capacity. The SELDI-TOF MS technology is advantageous for the detection of small prey proteins, many of which have an important biological function, for example, angiotensin II (1.3 kDa) or insulin (5.8 kDa). Small proteins and peptides can be overlooked when using conventional affinity capture techniques in combination

with gel electrophoresis analysis. On the contrary, one should be aware that large and/or highly glycosylated proteins are more difficult to identify by the SELDI-TOF MS technology where they often appear as broad peaks with a low intensity in the spectra.

SELDI-TOF MS detected biomarkers are only rarely identified, even if this would substantially increase the confidence in the results and would allow for the development of alternative immunoassay that could be easier to use in routine clinical practice. Unfortunately, on-chip proteolytic digestion and subsequent protein identification is not a realistic method since the biomarker is rarely pure on the spot and the SELDI-TOF instrument does not have a sufficient mass accuracy for the identification of the protein by peptide mass mapping. Therefore, to identify the protein corresponding to a particular SELDI peak, the protein should be

purified using chromatographic beads that carry functional groups similar to the chromatographic surface of the ProteinChip used for biomarker detection.

In order to isolate sufficient material for the identification of the interacting proteins we used IDM affinity beads, using the optimal conditions that had been established on the ProteinChip. Since several proteins bound to GlialCAM, a further purification step was required. In proteomic studies two techniques are most frequently used, 1- and 2-DE or RP-HPLC. We opted for the latter, taking advantage of LC where an extraction step is not required for protein isolation, increasing protein recovery and decreasing the risk of contaminating the samples. In addition, following LC, NP20 ProteinChips can be used to confirm the fractions containing the various interacting proteins according to their identified m/z , which might not be confirmed by the apparent protein masses observed by electrophoresis.

The choice to search for interacting partners to GlialCAM in serum from LPS stimulated mice came from the observation that preadministration of the extracellular domain of GlialCAM reduced the increase of the cytokine TNF α in this murine model. Using the novel SELDI-TOF MS based strategy to identify interacting proteins, we show that the extracellular domain of GlialCAM can form a complex with MBL-C and/or properdin, both of which have an immunomodulatory function. MBL is an activator of the lectin complement pathway and properdin is an activator of the alternative complement pathway [27, 28]. Both MBL and properdin play an important role in host defense and controlling bacterial (LPS) induced sepsis [29–31]. MBL-C levels in plasma is genetically variable and ranges between 20 and 10 000 ng/mL, with a further two- to three-fold increase during infection [32], making it an abundant plasma protein. In normal conditions properdin is less abundant, approximately 5–15 μ g/mL, but properdin levels are increased after LPS stimuli [33]. The increased expression of these proteins during infection correlates with the observation that the corresponding peaks are larger with plasma from LPS induced mice compared to plasma from normal mice. However, the high expression levels of the two identified proteins could raise the question whether we simply bound proteins that are abundant in LPS stimulated mice and indeed we did see a minor peak at approximately the same m/z as properdin in the spectra of the eluate from IL-18 immobilized beads. However, this peak was not present in the screening experiments using RS100 ProteinChips, which indicates that properdin or another protein with a similar molecular weight attaches with low affinity to the beads. We could not identify the protein causing this minor peak as the protein levels were too low. Furthermore, experiments in our laboratory show that GlialCAM interacts with the plant lectin concanavalin A (Con A) (unpublished data) and therefore the isolation of only two interacting proteins from a complex solution such as plasma where most proteins contain carbohydrate modifications indicated specific interactions.

In summary, we propose a new strategy for protein–protein interaction studies based on the SELDI-TOF MS technology. The extracellular domain of GlialCAM reduces the increase in TNF α levels associated with LPS induced immune response. The biological effect of the direct binding of GlialCAM to MBL-C and properdin, proteins involved in complement activation, requires further investigation. Together the method presented by us and that published by Lehmann *et al.* [9] demonstrate the powerful use of SELDI-TOF MS for protein interaction studies.

We are grateful to Christine Power and Alexandre Rolland for helpful discussions. We thank Patricia de Lys for developing the in vivo model. We acknowledge Laurence Friedli, Frederic Bollin, Holger Heine, and Thierry Battle for protein expression and Christian Arod, Jean-Philippe Gaudry, Pierre Graber, and Laurent Menoud for protein purification.

The authors have declared no conflict of interest.

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3. Discussion

When this study was originated, the only knowledge we had about GlialCAM was that it is composed of two Ig domains, a transmembrane domain and an intracellular domain, that it had homology with certain CAMs and that the pre-administration of the extracellular domain of GlialCAM reduces LPS induced TNF α response in mice. From there we started the conquest to identify the function of this novel protein.

In the first part of the thesis evidence is provided that GlialCAM is strongly and predominantly expressed in the human and mouse nervous system. In murine CNS, strong expression is found in various CNS regions like the cerebellum, the entorhinal cortex, the pons, the medulla and the spinal cord. *In vivo*, OLs were identified as the major cell type expressing GlialCAM. In addition, we also observed strong expression of GlialCAM in ependymal cells along the brain ventricles and the central canal of the spinal cord. A potential role of GlialCAM in myelination was supported by its temporal upregulation, correlated with MBP expression levels, during postnatal mouse brain development. *In vitro*, GlialCAM protein expression was detected in various developmental stages of the OL lineage. In A2B5 and O4 positive OLs, GlialCAM colocalizes with GAP43 in OL growth cone-like processes. GlialCAM expression was also observed in primary astrocytes. The GlialCAM amino acid sequence shares similarities with members of the CTX (cortical thymocyte marker in *Xenopus*) family of adhesion molecules including JAM-3 and ESAM (Chung et al., 2005). Members of the CTX family have been identified as trans-membrane components of tight junctions (TJ) between endothelial and/or epithelial cells in a wide range of tissues (Arrate et al., 2001; Eguchi et al., 2005; Hirata et al., 2001). Ependymal cells, which function as a protective and metabolic barrier between the CNS and CSF, are interconnected via numerous gap junctions, and some ependyma also express TJ associated proteins such as occludins and ZO-1 (Lippoldt et al., 2000; Petrov et al., 1994). Astrocytes do not form regular TJ in normal conditions but were shown to express the TJ associated protein claudin-1 under pro-inflammatory conditions, suggesting the formation of rudimentary TJs at astrocyte/astrocyte contacts during reactive astrogliosis (Duffy et al., 2000). OLs express TJ like structures under normal conditions. OSP/claudin-11, a major component of CNS myelin, is known to form TJ strands within myelin sheaths and has been proposed to have a structural role in myelin formation and maintenance (Gow et al., 1999; Morita et

al., 1999). The significant homology of GlialCAM to the CTX family together with its overall expression pattern indicates a potential function of the protein in the formation of TJ like structures in OLS, astrocytes and in the CNS ependyma.

In the second part of the thesis it is shown that, in the CNS, GlialCAM is regulated through phosphorylation, glycosylation and alternative splicing. Furthermore, we demonstrate that the protein is involved in important functions relating to OLS, including OPC migration, OL myelination and tumorigenicity.

Glycosylation is an important regulator of IgCAM stability and function. The carbohydrates attached to the protein backbone may regulate movement in the cell membrane thereby promoting interactions with membrane proteins on opposing cells. They may shield the protein from non-specific interactions and protect the protein from proteolytic degradation (Barclay, 2003). We demonstrate that GlialCAM is glycosylated in mouse brain and the results indicate that the carbohydrates protect the protein from degradation, since deglycosylation of GlialCAM leads to rapid degradation of the protein *in vitro*. However, other possible functions of the glycan structures attached to GlialCAM are still to be determined.

Adhesion molecules commonly form homo and/or hetero cis and trans interactions. In HEK293 cells, ectopic expression of GlialCAM did not induce GlialCAM cis-homo dimerisation but rather cell clustering indicating trans-homo interactions. GlialCAM homotypic trans interactions could be important for interactions of cell processes within the same cell or intercellular interactions, including between OLS, astrocytes, or OLS and astrocytes. GlialCAM might also form hetero-interactions. JAMs, which show close sequence homology with GlialCAM form both homophilic and heterophilic interactions (Mandell and Parkos, 2005). Extensive future investigations will be required in order to identify possible heterophilic interacting partners of GlialCAM and to identify possible sequential cis and trans interactions.

As mentioned above, GlialCAM is expressed in OL growth cone-like structures which have important functions in cell-cell interactions and migration. Hence, we decided to study whether GlialCAM plays a role in OL migration. We show that sGlialCAM together with PDGF-AA enhances OPC migration in the agarose drop assay. PDGF-AA induces cell surface receptor tyrosine kinase PDGFRA dimerisation and activation, leading to OPC proliferation and migration (Heidaran et al., 1993). The OPC migration promoting effect of sGlialCAM with PDGF-AA is not due to enhanced proliferation. Studies with

GlialCAM deficient OPCs demonstrate that the protein is not indispensable for OPC migration, on the contrary GlialCAM deficient OPCs show a tendency to migrate more than OPCs from WT littermates. This would indicate that GlialCAM could be involved in OPC-OPC adhesion rather than actual migration. Interactions between OLs are difficult to demonstrate but rare *in vitro* studies have shown such interactions. When OLs come into contact on the cell culture dish, the fine filopodia and lamellipodia of OL growth cone-like structures are retracted, eventually leading to growth cone collapse and inhibition of OL motility (Moorman, 1996). This suggests that OLs interact and respond to cell surface receptors on other OLs. Adding sGlialCAM could disrupt OPC interactions in the agarose drop migration, leading to a change in OPC morphology, possibly enhanced processes and growth cone-like structures, which allows PDGF to induce motility.

Preliminary studies on CNS myelination in GlialCAM deficient mice show reduced MBP and CNPase levels, and vacuoles in the anterior commissure white matter tracts. This could suggest a role for GlialCAM in myelin tight junctions, since similar myelin abnormalities have been observed in double knockout mice for PLP and OSP/Claudin-11 or GalC/sulphatide (Chow et al., 2005; Coetzee et al., 1999). In addition, in mice deficient for the large MAG isoform the most common myelin defect was the retention of cytoplasm within the myelin lamella, suggesting a delayed compaction process (Fujita et al., 1998). Remyelination is a common feature of early stage multiple sclerosis (MS), however with repeated or prolonged episodes of demyelination during the course of the disease, the inherent capacity of the CNS to remyelinate becomes exhausted and the axon remains demyelinated and becomes vulnerable to atrophy. OPCs in the adult CNS are believed to be the major source of remyelinating OLs (Levine et al., 2001). In animal models of demyelination, PDGF-AA administration enhances remyelination (Allamargot et al., 2001; Vana et al., 2007). The mechanisms by which PDGF-AA improves remyelination could include OPC proliferation, survival and migration. Taken the promigratory effect on OPCs of sGlialCAM in conjunction with PDGF-AA and the need for OPC migration to the site of myelin damage in demyelinating diseases, such as MS, it could be interesting to study the effect of sGlialCAM with PDGF-AA on CNS remyelination.

Since GlialCAM expression was shown to be downregulated in hepatocarcinoma and we found the protein is mainly expressed in the central nervous system we examined its

expression in oligodendroglioma and astrocytoma (Chung et al., 2005). We found that GlialCAM is downregulated in human grade II and III oligodendrogliomas and grade I and IV astrocytomas. Gliomas of all grades are highly invasive CNS tumours and the downregulation of GlialCAM in these tumours could support our hypothesis that loss of GlialCAM reduces OPC-OPC adhesion and hence, promotes motility. Downregulation of other IgCAMs, such as NCAM and CAR, has also been reported to enhance tumorigenicity and cell invasion (Cavallaro and Christofori, 2004; Kim et al., 2003; Fuxe et al., 2003). Current data indicate that loss of NCAM results in defective cell-matrix adhesion which leads to cell detachment and invasion, while loss of CAR leads to decreased microtubule stability and a transition from a quiescent to an invasive, mesenchymal phenotype. Further studies are required to determine the role of GlialCAM in tumorigenicity. GlialCAM is presumably downregulated in gliomas through DNA hypermethylation, since incubation of a A-172 human glioblastoma cell line with an inhibitor of DNA methylation, 5-azacytidine, enhances GlialCAM expression levels in these cells. DNA methylation is the most common epigenetic variation in human cancers (Kanai and Hirohashi, 2007). Several tumour cells have been shown to have genome wide hypomethylation with regional hypermethylation and tumour suppressor genes are commonly silenced through hypermethylation. Examples of CAMs that are silenced through hypermethylation in human tumours, include E-cadherin and NeCl-2 (Yoshiura et al., 1995; Heller et al., 2006). Hypermethylation of GlialCAM could be used as a marker for glioma tumorigenicity (Kanai and Hirohashi, 2007). As described in the introduction, there is a lack of reliable markers for gliomas, especially oligodendrogliomas (ODs). The most robust objective marker for OD today is the allelic loss of the 1p and 19q loci, while histological analysis of tumour samples is subjective to the investigator. Together with a panel of other markers, changes in GlialCAM expression could be used to identify gliomas. Furthermore, GlialCAM gene-specific demethylation agents could be of therapeutic value in the treatment of gliomas.

The present study demonstrates for the first time that GlialCAM is phosphorylated, not only following ectopic expression in HEK cells but also in primary rat astrocytes. Furthermore, *in vitro* studies show that the purified intracellular domain of the protein can be phosphorylated by ERK2 and ASK1. Phosphorylation could be an important feature for the regulation of GlialCAM function and the intracellular domain contains several potential phosphorylation sites. ERK2 mediated GlialCAM phosphorylation could be related to the pro-migratory function observed in OPCs after concomitant incubation with

PDGF-AA, since PDGFR stimulation induces sustained ERK activation leading to cell motility (Silletti et al., 2004). In adult neural progenitor cells that have the potential to give rise to neurons, astrocytes and OLs, ectopic expression of ASK1 promotes neuronal differentiation, while glial cell development is inhibited (Faigle et al., 2004). Taking into account that ASK1 inhibits glial differentiation and that GlialCAM is expressed in all stages of OL development and in astrocytes, one could hypothesise that GlialCAM phosphorylation by ASK1 has an inhibitory effect on GlialCAM function.

In the third part of the thesis we show that sGlialCAM administration before LPS, lowers LPS induced TNF α levels in a mice animal model. In order to determine the mechanism of sGlialCAM in this *in vivo* model, we isolated and identified from the plasma of LPS stimulated mice, two proteins, mannose binding lectin C (MBL-C) and properdin, which interact with sGlialCAM. For this purpose we developed a novel strategy for the detection and identification of protein binding partners. This encompassed using ProteinChips and SELDI-TOF MS to detect protein–protein interactions. Followed by a robust method for isolating and identifying proteins detected with the SELDI-TOF MS, using affinity beads, RP-HPLC, nano LC MS/MS and bioinformatic tools. It is one of the first reports of successfully using the SELDI-TOF MS method to identify protein-protein interactions from complex biological samples.

Properdin is a soluble glycoprotein that positively regulates the alternative complement pathway (Fearon and Austen, 1975). The alternative complement pathway can be activated either independently or following activation of the classical or lectin pathway. A recent study demonstrates that properdin can bind directly to microbial targets and is indispensable for LPS induced complement activation (Kimura et al., 2008). MBL is an evolutionary conserved host defense protein that activates the lectin complement pathway. It has been well established that MBL binds to LPS and that the binding mediates complement activation (Brade and Brade, 1985; Swierzko et al., 2003). A recent report shows that following LPS stimulation, the complement system is directly involved in regulating TLR4 induced cytokine production and release (Zhang et al., 2007). Hence, sGlialCAM binding to properdin and MBL-C could prevent them from activating the complement pathway and thereby decrease complement enhanced TLR response to LPS, leading to decreased secretion of TNF α . However, further investigations are required to determine the role of sGlialCAM in complement activation. Whether soluble forms of GlialCAM exist naturally still remains to be determined,

nevertheless the recombinant soluble form of the protein could be of therapeutic value in treating septic shock.

Taken together, the results presented in this thesis suggest a role of GlialCAM as both an adhesive and signalling molecule in OLs and astrocytes. It seems to play a functional role both in early stage OPCs as well as mature myelinating OLs. Furthermore, it could act as a tumour suppressor gene in oligodendroglioma and astrocytoma. Moreover, sGlialCAM presumably reduces LPS induced immune response in mouse plasma by binding to MBL-C and properdin, and thereby preventing their function to induce complement activation.

The overall goal of our studies was to identify molecules with therapeutic potential. Extensive studies are still required to characterise GlialCAM, however our data suggest several potential lines of investigation. sGlialCAM could have a potential therapeutic value as a inducer of OPC motility in conjunction with PDGF in demyelination pathologies or an inhibitor of complement activation following LPS induced dysregulation of the immune system. Furthermore, therapeutics targeting the processes that cause GlialCAM downregulation in oligodendroglioma and astrocytoma could be of value. The results presented in this thesis provide a foundation for future research on GlialCAM and its possible therapeutic potential.

4. Concluding remarks

The work presented in this thesis demonstrates the following about GlialCAM:

Tissue expression level

- GlialCAM is mainly expressed in the CNS in both humans and mice, with the strongest expression observed in white matter regions and the lining of the ventricles of the brain, and the lining of the central canal of the spinal cord. It is also expressed in isolated sciatic nerve. We detected low level of GlialCAM expression in human liver, while none was detected in mouse.
- Two GlialCAM isoforms are detected in human and mouse brain extracts, presumably splicevariants.
- The GlialCAM isoforms are differentially regulated during post-natal development and the expression correlates with MBP expression levels.

Cellular expression level

- In purified rat primary CNS cells, GlialCAM is expressed in oligodendrocytes and astrocytes. In both cell types, the protein is expressed at the cell body as well as at the tip of and along processes extended by these cells. In Oligodendrocytes, GlialCAM expression co-localises with GAP-43, a marker for oligodendrocyte growth cone-like structures. In cultured, GlialCAM was detected in different differentiation stages from oligodendrocyte precursor to mature oligodendrocytes. GlialCAM expression in the lining of the ventricles and the central canal suggests its expression in ependymal cell.

Posttranslational modifications

- GlialCAM is phosphorylated in cultured astrocytes and ERK2 and ASK1 phosphorylates GlialCAM intracellular domain *in vitro*.

- GlialCAM is glycosylated in mouse brain and deglycosylation leads to its rapid degradation.

Functions

- GlialCAM forms trans-interactions, while cis-dimerisation is not detected.
- Treating OPCs with sGlialCAM enhances their migration, presumably due to reduced GlialCAM mediated contact between OPCs. This is supported by the fact that GlialCAM deficient OPCs show enhanced migration compared to WT controls.
- Mice deficient in GlialCAM show reduced levels of myelin associated proteins, MBP and CNPase, and vacuolisation within white matter tracts.
- GlialCAM is downregulated in oligodendrogliomas and astrocytomas, presumably through DNA hypermethylation.
- In plasma from LPS stimulated mice, sGlialCAM interacts mainly with two proteins, mannose binding lectin C (MBL-C) and properdin. Sequestration by sGlialCAM of these two LPS promoted inducers of complement activation could explain the observed reduction in TNF α levels.

A novel strategy for the detection and identification of protein binding partners was developed during the course of the work

- The method combines ProteinChips and SELDI-TOF MS with more conventional proteomics tools, affinity beads, RP-HPLC, nano LC MS/MS and bioinformatics. It is one of the first reports of successfully using the SELDI-TOF MS method to identify protein-protein interactions from complex biological samples.

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5. Annex

During the course of my PhD, I participated in research projects that were not included in this thesis, but gave rise to the publication included below.

Research paper

Quantitative detection of therapeutic proteins and their metabolites in serum using antibody-coupled ProteinChip® Arrays and SELDI-TOF-MS

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Received 12 June 2006; received in revised form 31 August 2006; accepted 4 October 2006

Available online 2 November 2006

Abstract

One of the important steps in developing protein therapeutics is the determination of their preliminary PK *in vivo*. These data are essential to design optimal dosing in animal models prior to progressing to clinical trials in man. The quantitative detection of protein therapeutics in serum is traditionally performed by ELISA, which has the prerequisite of the availability of the appropriate monoclonal antibodies. We have developed an alternative method using polyclonal antibodies immobilized on ProteinChip Arrays and SELDI-TOF mass spectrometry. This method has an advantage over ELISA since it provides simultaneously information on the clearance rate of the protein and its *in vivo* processing. We compared these two methods using a RANTES variant, [⁴⁴AANA⁴⁷]-RANTES as the test protein in this study. Using SELDI-TOF mass spectrometry, we were able to establish that the protein is readily oxidized in serum, and moreover is processed *in vivo* to produce a truncated 3–68 protein, and undergoes a further cleavage to produce the 4–68 protein. These modifications are not identified by ELISA, whilst the serum exposure profiles determined by the two methods show essentially similar protein concentration values.

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Keywords: Protein processing *in vivo*; SELDI; RANTES; Pharmacokinetics

1. Introduction

Protein therapeutics or biologicals play a major role alongside small molecule therapeutics in treating many diseases, and in fact many strategies using biologicals are now replacing or supplementing therapies such as steroids that have been used for decades. Protein therapeutics are generally applied to interactions that are not easily targeted by small molecules, the most common example being the interaction between cytokines and their receptors (Johnson-Leger et al., 2006). In the immune system for example, the interaction of cytokines, interleukins and growth factors with their receptors

Abbreviations: SELDI-TOF-MS, Surface Enhanced Laser Desorption Ionization time-of-flight mass spectrometry; MALDI, matrix-assisted laser desorption/ionization; PK, pharmacokinetic; GAG, glycosaminoglycan; RANTES, Regulated on Activation, Normal T Expressed and Secreted; CCL5, chemokine (CC motif) ligand 5.

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involve large surfaces, which are not amenable to blockade by small molecules, but can be inhibited by binding proteins or neutralizing antibodies. Natural agonists have similarly given birth to their own class of protein therapeutics: the activation of cytokine-, interleukin-, and growth factor-receptors often requires activation that may be best achieved with the endogenous ligand, and many such therapies are on the market, including interleukin-1 α for Hepatitis-C infection, interferon- β for multiple sclerosis, and the biggest block buster in the protein therapeutic field, Epoetin alpha for chronic renal failure.

An essential step in the development of protein therapeutics is the determination of their bioavailability by various routes of administration such as either intravenous (i.v.) or subcutaneous (s.c.) suitable for injection in man, or the intraperitoneal (i.p.) route for testing of efficacy in animal models. Classically this is determined by the ELISA technique, but this methodology is time consuming to develop since it first requires the production of suitable antibodies, ideally monoclonals. Secondly, problems of species cross-reactivity are often a hindrance since ELISAs are required to identify the candidate therapeutic protein in a number of species during the pre-clinical development.

To develop an alternative method we used a variant of RANTES/CCL5 with targeted amino acid mutations in its principal GAG binding site (Proudfoot et al., 2001). RANTES/CCL5 is a member of the chemokine family of proteins, which play an important role in leukocyte trafficking and homing (Schall et al., 1990). RANTES mediates its effects via several 7 transmembrane G protein-coupled chemokine receptors, CCR1, CCR3, and CCR5 (Pakianathan et al., 1997). We have demonstrated that in addition to the well characterized interaction that RANTES has with its receptors, a second, less well understood interaction with immobilized glycosaminoglycans appears to be essential in order for RANTES to mediate its chemotactic effects *in vivo* (Proudfoot et al., 2003). Furthermore, this variant has been shown to inhibit the recruitment capabilities of RANTES *in vivo*, an observation which has been extended to show amelioration of disease in experimental allergic encephalomyelitis (EAE), a mouse model of multiple sclerosis (MS) (Johnson et al., 2004). In order to further understand this inhibitory capacity we used both an ELISA as well as tracing of iodinated proteins and showed that differences in *in vivo* distribution over time following administration are directly related to the GAG binding properties of the protein, i.e. RANTES remains essentially at the local site of injection for a period of up to 4 h, whereas [44 AANA 47]-RANTES

rapidly enters the bloodstream and is readily detectable in the serum as early as 30 min post-dose. However, the ELISA does not allow distinction between RANTES and its variant (Johnson et al., 2004).

Surface Enhanced Laser Desorption Ionization time-of-flight mass spectrometry (SELDI-TOF-MS) enables multiple protein analyses on a single experimental platform, combining selective protein capture with sensitive and quantitative mass spectrometric analysis. The experimentally measured mass of the captured molecules allows for the accurate detection of multiple variants of a given protein in a single assay (Rossi et al., 2006). Also, nonspecific antibody cross-reactivity, that might be present in an ELISA, is eliminated using the SELDI, as only the peaks with the accurate molecular mass are quantified. Furthermore for capture from biological samples, polyclonal antibodies are most suitable, and moreover are relatively rapidly produced. We therefore used this technology to distinguish between wild type RANTES and the [44 AANA 47]-RANTES variant.

Using serum samples from mice dosed with [44 AANA 47]-RANTES analyses by ELISA and SELDI technology yielded similar pharmacokinetic profiles. Additionally, as well as demonstrating that SELDI may be used as an alternative to ELISA, we show by changes in mass that the protein is processed *in vivo*. Here we show that not only was the protein truncated at the amino terminus to produce first the 3–68 form, and subsequently a further truncation produced the 4–68 form, but also that on exposure to serum, the protein was rapidly oxidized. It has been previously demonstrated that chemokines are vulnerable to a variety of proteolytic processing activities (Struyf et al., 1998; Proost et al., 2001; Overall et al., 2002).

Thus determination of the plasma exposure of candidate protein therapeutics proteins by SELDI will not only rapidly provide information on the PK properties of the protein, but can also provide precious information on *in vivo* processing as well as biochemical modifications such as oxidation – information that is crucial for the development of protein therapeutics.

2. Materials and methods

2.1. Instrumentation

SELDI-TOF-MS, Surface Enhanced Laser Desorption Ionization time-of-flight mass spectrometry a technology supplied by Ciphergen Biosystems (Bruenner et al., 1996; Davies et al., 1999) was performed using a Biology System IIc (Ciphergen Biosystems Inc., Fremont, CA).

2.2. Reagents

Polyclonal anti-human RANTES antibodies (purified) for SELDI were purchased from PeproTech EC Ltd (London, UK) and R&D systems (Abingdon, UK). Rabbit anti-human RANTES (purified and biotinylated) for ELISA were purchased from Pharmingen (Jacksonville, USA). Streptavidin-HRP conjugate was purchased from Zymed (Basel, Switzerland). Ethanolamine was purchased from Sigma. TritonX-100, Urea, Sodium chloride, Tris, Hepes and sinapinic acid were purchased from Fluka (Basel, Switzerland). Chaps was purchased from Boehringer Mannheim GmbH. α -cyano-4-hydroxycinnamic acid (CHCA) was purchased from Ciphergen Biosystems Inc (California, USA). RANTES and [44 AANA 47]-RANTES were prepared as previously described (Proudfoot et al., 2003).

2.3. In vivo sampling.

8 week old female BALB/c mice (Janvier, France) were injected with 10 μ g of [44 AANA 47]-RANTES in 200 μ l sterile PBS via the intra-peritoneal (i.p.) route at t 0. Terminal bleeds via cardiac puncture were obtained from 3 mice per time point. Blood was collected into Microtainer serum separator tubes (Becton Dickinson, USA) and serum harvested. Serum samples were stored at -80°C until further analysis.

2.4. Sandwich ELISA for protein detection

96 well Nunc-ImmunoTM MaxiSorpTM surface plates (Nalge Nunc International, Denmark) were coated with 50 μ l per well of a rabbit anti-human RANTES purified antibody at 4 μ g/ml in a 2 M sodium bicarbonate buffer and incubated overnight at 4°C . Following a wash and blocking step, serum samples were added at 100 fold dilutions in PBS containing 1% BSA and 0.05% TWEEN-20. Subsequent 2 fold dilutions of each sample were made for a total of 8 dilutions. Measurements were made in duplicate for each sample. Standards were prepared with [44 AANA 47]-RANTES in PBS containing 1% BSA and 0.05% TWEEN-20 at concentrations ranging from 2000–31.25 pg/ml. Normal mouse serum was added to the standards at a 100 fold final dilution. Following a 3 h incubation at room temperature the plate was washed and 100 μ l of a 4 μ g/ml solution of rabbit anti-human RANTES biotinylated antibody was added to each well, and incubated for 1 h at room temperature. The plate was washed and Streptavidin-HRP conjugate was added to each well and incubated for 30 min at room temperature. The plate was washed and substrate

solution containing *o*-phenylenediamine dihydrochloride (OPD) and H_2O_2 was added to each well. The plate was kept in the dark for 20 min. The reaction was stopped with 50 μ l per well of 20% H_2SO_4 , and the optical density (OD) of each well was determined using a Multiskan spectrophotometer (Labsystems, Basel, Switzerland), set to wavelength 490 nm with wavelength correction set to 570 nm and data analyzed with Excel (Microsoft, CA, USA). The ELISA showed no cross reactivity for murine RANTES.

2.5. Antibody selection and SELDI TOF-MS set-up

The quality of commercial polyclonal anti-RANTES antibodies (PeproTech, London, UK and R&D systems, UK) was controlled by SELDI-TOF-MS, using the ProteinChipTM Biology System Ilc (Ciphergen Biosystems Inc., Fremont, CA) and the normal phase, NP20 ProteinChipTM Arrays. For this purpose a dilution series of 1/1, 1/2, 1/3, 1/4 of the antibodies was prepared in 50 mM NaHCO_3 , pH 9.0. 5 μ l of each solution was added to respective spots on the NP20 ProteinChip Array. The spots were washed twice with 5 μ l deionised water and then 0.8 μ l of saturated sinapinic acid (Fluka, Switzerland) was dried onto the spots. The mass analyzer was calibrated using the commercial All-in-1 protein standard mix provided by Ciphergen Biosystems.

2.6. Preparation of antibody-coupled ProteinChip Array

5 μ l of 1 mg/ml anti-RANTES antibody (PeproTech, London, UK) diluted 1/3 in 50 mM NaHCO_3 pH 9.0 was applied to each spot on RS100 ProteinChipTM Arrays (Ciphergen Biosystems Inc., Fremont, CA) and incubated for 2 h at room temperature in a humidified chamber. Nonspecific binding sites were blocked with 0.5 M ethanolamine/PBS for 1 h at room temperature. The arrays were washed 3 times for 5 min with PBS containing 0.1% Triton X-100 and used on the same day for analyses.

2.7. Standard curve

The standard curve was generated by diluting [44 AANA 47]-RANTES to 40, 32, 26, 20, 14, 8, 4, 2 and 0 ng/ml in BALB/c serum, respectively. The solutions were further diluted 1/1 in PBS containing 0.1% Triton X-100 and 50 μ l was added to RS100 ProteinChip Array spots that had been prepared with the anti-RANTES antibody. The standard curve was generated in parallel with the sample analyses and processed and analyzed identically, following the description below.

2.8. Serum analyses

The serum samples were diluted in BALB/c mouse serum, where the dilution factors were estimated from the concentrations measured by the ELISA, so that the [$^{44}\text{AANA}^{47}$]-RANTES protein concentration remained within the range (50–1000 pg protein) of the standard curve. The diluted samples were further diluted 1/1 in PBS, 0.1% Triton X-100, before incubating for 2 h at room temperature on RS100 protein array spots that had been pre-incubated with anti-RANTES antibody. The arrays were then washed twice for 5 min with PBS containing 0.1% Triton X-100 and once for 3 min with 50 mM Tris pH 9.0 containing 1 M Urea, 2% CHAPS and 0.5 M NaCl. They were then rinsed with 5 mM HEPES pH 7.2 for 15 s at room temperature. The matrix, α -cyano-4-hydroxycinnamic acid, was dried onto each spot on the array and mass analysis was performed by SELDI-TOF-MS, using the ProteinChip™ Biology System IIc. Spectra were generated using an automated protocol and a positive ion mode with a laser intensity of 150, a sensitivity of 5 which are the manufacturer-defined settings specific to this type of instrument, and several conditions were tested before selecting the most appropriate parameters. The mass focus was set between 2–20 kDa. In order to obtain quantitative data the mass spectra from each spot were accumulated from 155 laser shots over 31 different and non-overlapping positions.

2.9. SELDI-TOF-MS data analysis

Spectra of standards and samples were generated using an identical automated protocol allowing us to merge the spectra into the same file and treat them

identically for analysis. The mass analyzer was calibrated using the all-in-1 peptide standard (Dynorphin 2147.50 Da, ACTH 2933.50 Da, Bovine insulin b-chain 3495.94 Da, Human insulin 5807.65 Da, Hirudin 6963.5 Da) from Ciphergen Biosystems Inc, following the manufacturers' protocol. Peaks in the spectra obtained with Ciphergen's technology platform and Proteinchip software were annotated using the average neutral mass of the peptide or protein followed by their protonated charge state. Normalization of signal intensities of all spectra was carried out using a serum peak with a mass of 8120 Da as a reference. This normalization procedure produced the most accurate results and was used for the production of the standard. This serum peak, present in all samples, and the peak of [$^{44}\text{AANA}^{47}$]-RANTES (7620 Da, measured on a Voyager DE-PRO Maldi-ToF mass spectrometer, Applied Biosystems, Foster City, CA 94404, U.S.A.) were used as internal standards for exact alignment of the spectra around the mass range of interest. The height and area of peaks observed for [$^{44}\text{AANA}^{47}$]-RANTES at 7620 Da (full-length), 7436 Da (truncated) and 7273 Da (truncated) were measured. For all standards and samples analyses were performed in triplicate. Values used in our graphs are the sum of the values obtained for the un-oxidized and oxidized peaks.

3. Results

3.1. RANTES ELISA

In order to investigate the PK profile of [$^{44}\text{AANA}^{47}$]-RANTES an ELISA was used consisting of a commercially available anti-human RANTES antibody pair.

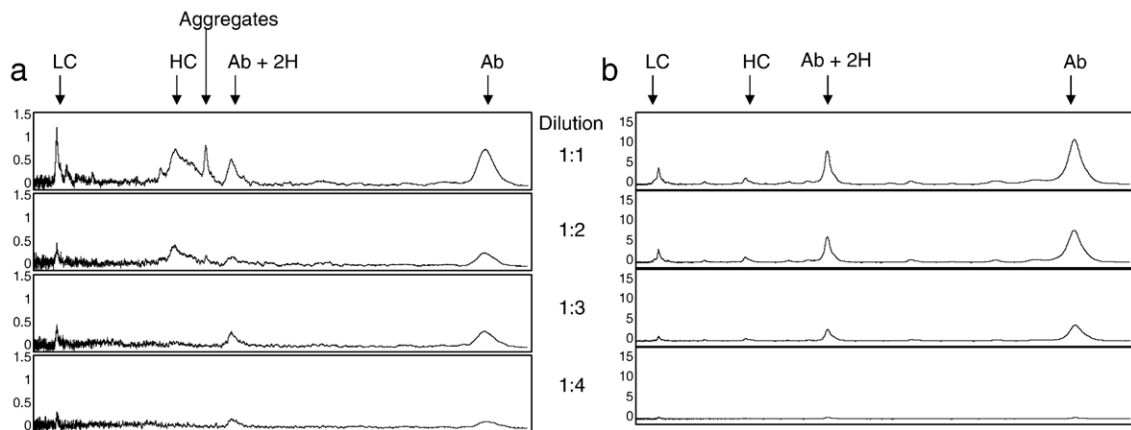


Fig. 1. Comparison of commercially available polyclonal anti-RANTES antibodies. A dilution series 1/1, 1/2, 1/3, 1/4 (from top to bottom) in 50 mM NaHCO_3 of R&D systems (a) and Peprotech (b) anti-human RANTES antibodies were analyzed on NP20 ProteinChip Arrays before starting RS100 ProteinChip Array experiments. Ab; intact antibody, Ab+ 2H; double-charged antibody, HC; Antibody heavy chain, LC; Antibody light chain.

This ELISA was unable to detect murine RANTES from serum, but was able to detect both human RANTES and [$^{44}\text{AANA}^{47}$]-RANTES in serum from mice following injection with the proteins. This ELISA was used to track the distribution of human RANTES and [$^{44}\text{AANA}^{47}$]-RANTES following i.v. or i.p. injection into mice (Johnson et al., 2004). Both RANTES, and [$^{44}\text{AANA}^{47}$]-RANTES can be detected in the serum of mice following i.v. administration up to 4 h following injection, after which levels of [$^{44}\text{AANA}^{47}$]-RANTES were no longer detected. Following i.p. adminis-

tration, [$^{44}\text{AANA}^{47}$]-RANTES was detectable at high levels in the serum, with a peak occurring at 30 min after injection.

3.2. Antibody selection for SELDI analysis

Two sources of commercial polyclonal antibodies were investigated for the experiment. At first the antibodies were submitted to a quality control using the SELDI-TOF MS technology. Analysis of four dilutions of equal amounts of each antibody on NP20 protein

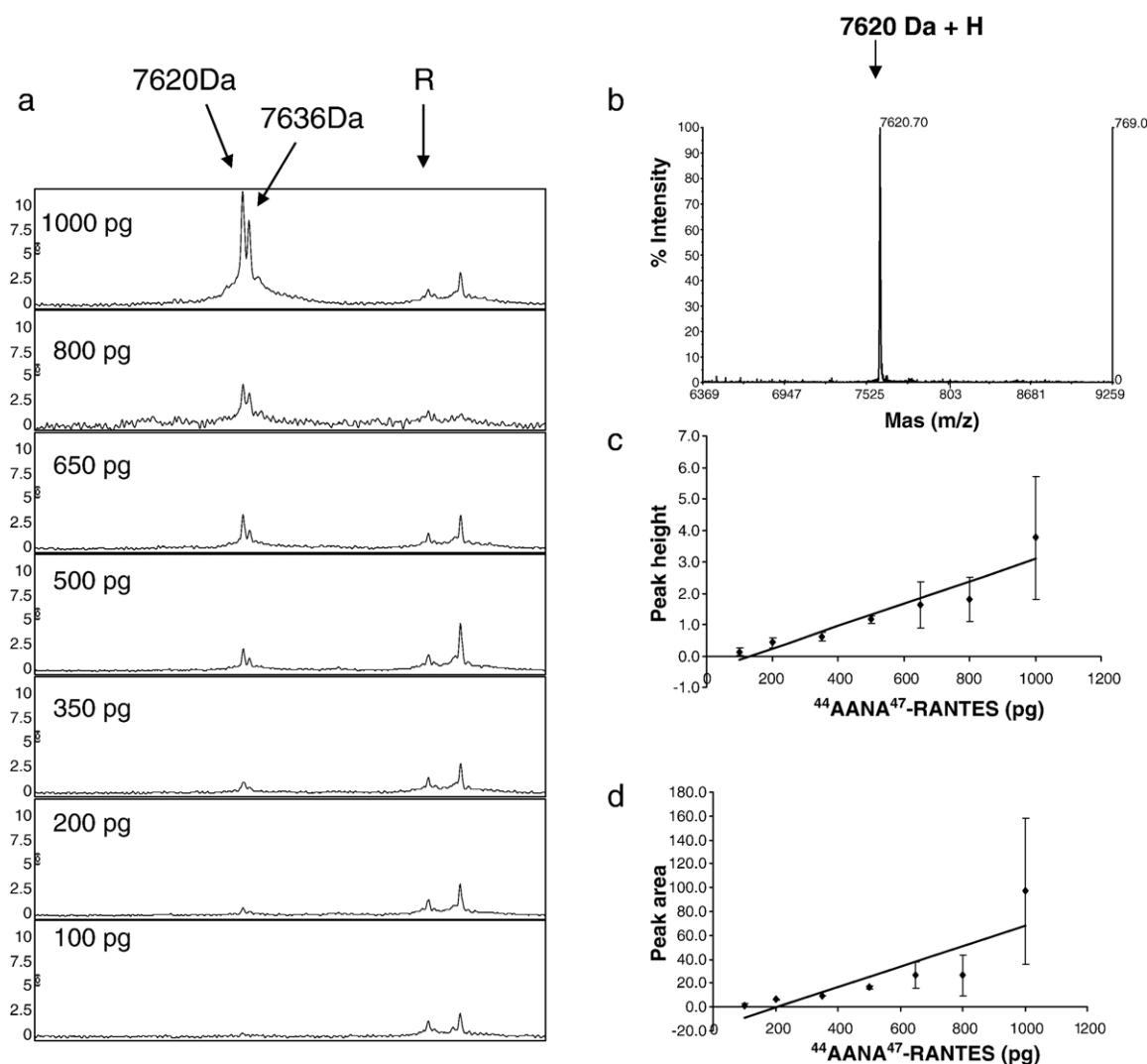


Fig. 2. Standard curve of purified human [$^{44}\text{AANA}^{47}$]-RANTES in mouse serum. (a) A dilution series of purified human [$^{44}\text{AANA}^{47}$]-RANTES in mouse serum was generated and analyzed on the RS100 ProteinChip Array using an anti-RANTES antibody as bait. The antibody does not detect the murine variant of RANTES. In contact with serum [$^{44}\text{AANA}^{47}$]-RANTES undergoes oxidation (7636 m/z peak). (b) Oxidation is not observed in the protein dissolved in PBS. For comparison, standard curves were generated using both the peak height (c) and area (d). The values of the unoxidized and oxidized protein were combined for each condition. The spectra were normalized to a nonspecific serum peak, R, at 8120 Da. Only peaks with a signal-to-noise value above 3 were considered. The lowest observed amount is 200 pg protein.

arrays showed that the PeproTech antibody gave a cleaner and more intense signal than the R&D antibody as shown in Fig. 1. (R&D subsequently spontaneously informed us that this particular lot was of poor quality and contained insoluble protein upon re-constitution as is seen in Fig. 1a). Furthermore the 1/3 dilution allowed the pH to be changed from that of the PBS solution in which the antibody was supplied to that of 9.0 which is required for coupling of the antibody to the RS100 arrays, and still produced a signal of sufficient intensity (Fig. 1b).

3.3. SELDI-TOF-MS PK analysis

The serum samples analyzed by ELISA were further analyzed using the SELDI-TOF-MS technology. The aim was to assess whether the two techniques were comparable and also to take advantage of the more detailed information that can be obtained by the latter. To generate a standard curve, the anti-RANTES antibody was coupled to pre-activated ProteinChip Arrays (RS100) and a dilution series of [$^{44}\text{AANA}^{47}$]-RANTES in mouse serum was applied. The ProteinChip Arrays were treated according to manufacturer's instructions.

The mass analyzer was calibrated using the all-in-one-peptide standard provided by Ciphergen Biosystems. Calibrated spectra were further aligned using the peak of [$^{44}\text{AANA}^{47}$]-RANTES (7620 Da) and the peak of a nonspecific mouse serum protein (8120 Da). As shown in Fig. 2 [$^{44}\text{AANA}^{47}$]-RANTES was specifically captured by the antibody, the mass of 7620 Da corresponds to its theoretical mass (the 4 cysteine residues are linked by two internal disulfide bonds). The nonspecific mouse serum protein was used as a reference for the normalization of signal intensities of [$^{44}\text{AANA}^{47}$]-RANTES and its metabolites measured in serum. This procedure produced the most accurate results. A common procedure used in SELDI biomarker discovery experiments consists in using the Total Ion Current (TIC) for normalization of signals. This normalization method works well when the spectra contain a large collection of peaks. In the present study the spectra contained only a few nonspecific peaks in addition to the peaks of the protein of interest and normalization to the TIC would have generated inaccurate results. The amount of capture antibody cross-linked on the surface of the different spots might vary between spots. These variations will affect the amount of antigen captured and the intensity

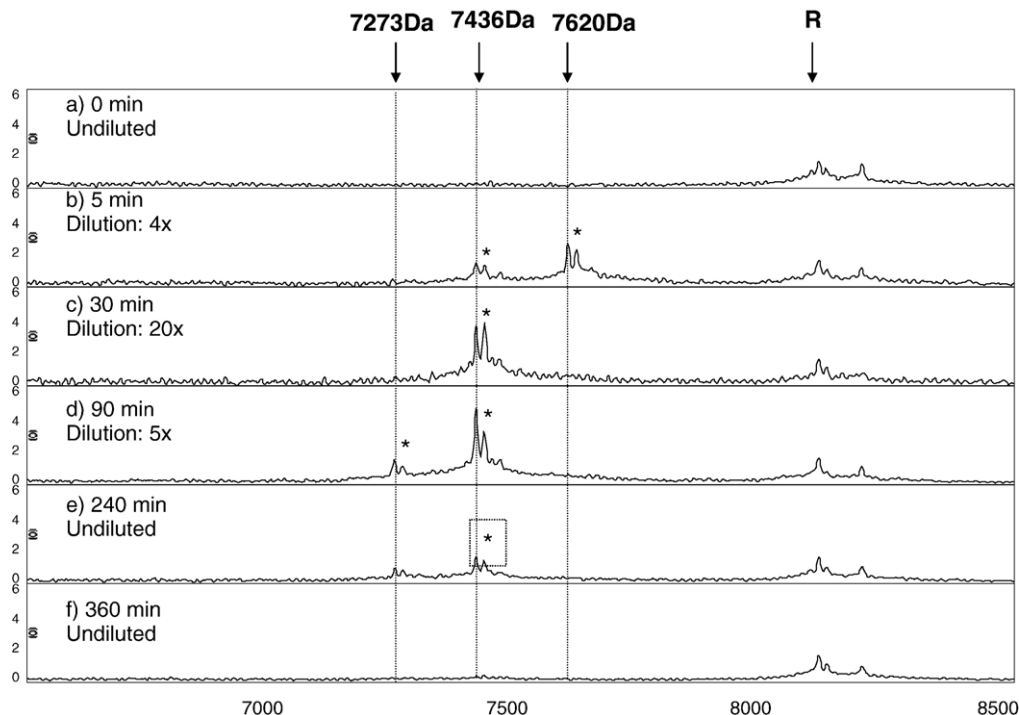


Fig. 3. Truncation of human [$^{44}\text{AANA}^{47}$]-RANTES *in vivo*. Serum was collected from mice at specific time points after the administration of human [$^{44}\text{AANA}^{47}$]-RANTES (0.5 mg/kg i.p.). The serum samples were diluted so that the protein concentration could be quantified using the standard curve. The samples were analyzed on the RS100 ProteinChip Array using the same procedure as that for the standard curve. Each time point is a representation of three independent mice. The spectra were normalized to a nonspecific serum peak, R, at 8120 Da.

of the signal measured by SELDI. The nonspecific 8120 Da serum protein is captured by our antibody and the intensity of its signal varies according to the amount of antibody on the spots. Using this protein for normalization corrects for the differences between spots and therefore increases the accuracy of our measurements. It is very unlikely that the same protein with a mass of 8120 Da is detected in sera from other species, therefore the protocol presented here works only for mouse sera. The selected antibody did not detect the murine (endogenous) protein. In accordance with pre-

viously published data by Lim et al. showing that RANTES is readily oxidized in PBMC culture supernatants, we also observed that the protein is oxidized in serum, rendering two RANTES specific peaks on the SELDI spectra (a 7620 Da peak representing the unoxidized and a 7636 Da peak representing the oxidized protein). Fig. 2b shows that RANTES had not undergone oxidation before contact with serum. Both the peak of the oxidized and the unoxidized protein were accounted for in the standard curve. Each condition was done in triplicate. Also, both peak intensity (Fig. 2c) and area

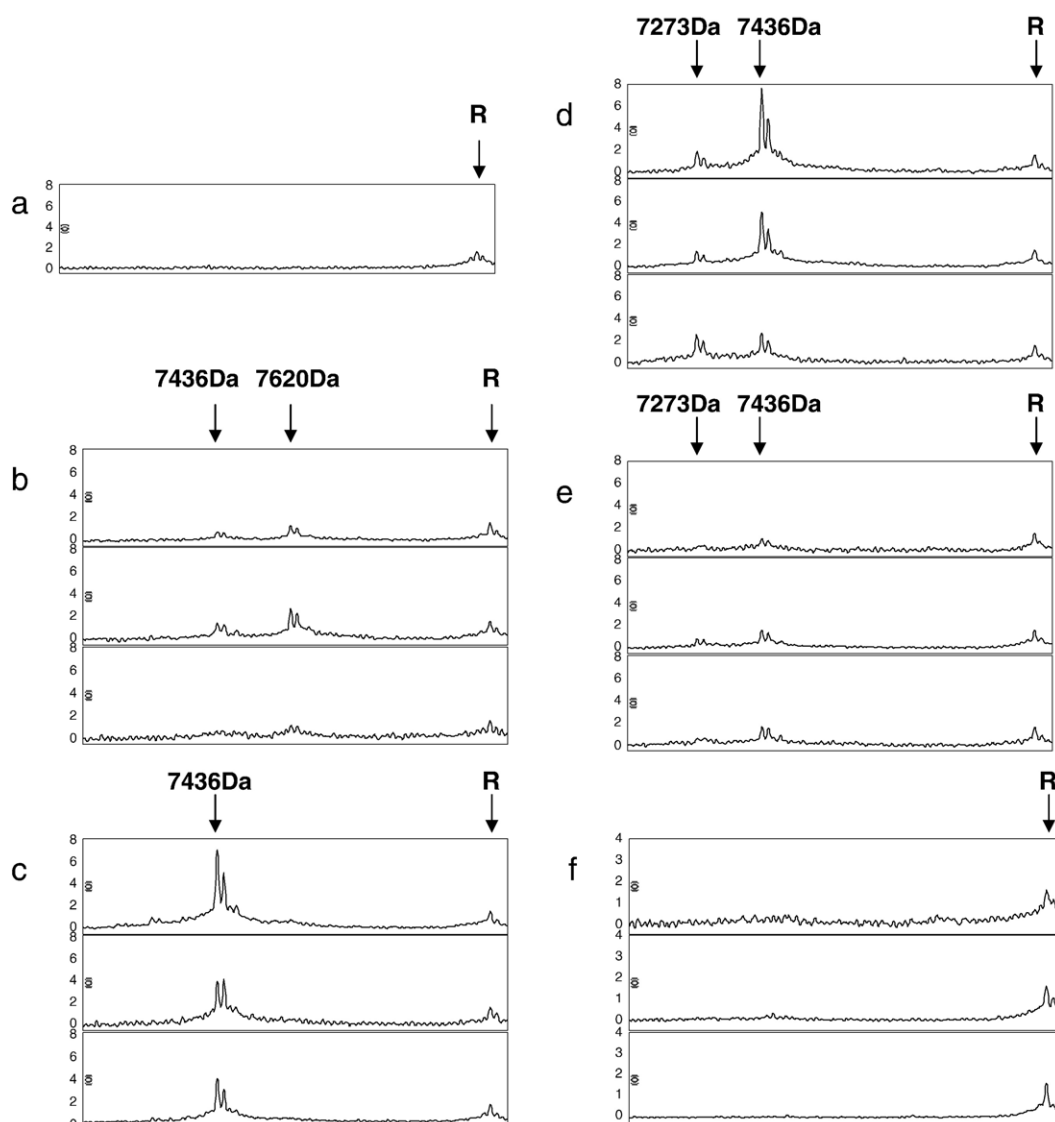


Fig. 4. Comparison of the truncation of [$^{44}\text{AANA}^{47}$]-RANTES in three independent mice. The concentration and digestion pattern of [$^{44}\text{AANA}^{47}$]-RANTES in serum 0 min (a), 5 min (b), 30 min (c), 90 min (d), 240 min (e) and 360 min (f) post i.p. injection was studied in three mice in parallel to test for differences between individuals. Each spectrum is a representation of three spectra produced for one condition. The spectra were normalized to a nonspecific serum peak at 8120 Da (R).

(Fig. 2d) were evaluated with the purpose to determine whether the two measurements gave similar results. Accordingly, both methods gave similar standard curves with a comparable slope, however the correlation coefficient R^2 was higher when measuring peak intensity ($R^2=0.9$) than area ($R^2=0.7$).

Serum samples of BALB/c mice injected intraperitoneally (i.p.) with [$^{44}\text{AANA}^{47}$]-RANTES were analyzed at various time points after the injection. The analysis of the samples was performed identically to that of the standard curve. The samples were diluted according to the concentration estimated with ELISA so that the intensities remained within the range of the standard curve. The spectra were normalized together with the spectra of the standard curve to a nonspecific serum peak. The results showed that, as already demonstrated by ELISA, maximal detection of [$^{44}\text{AANA}^{47}$]-RANTES in serum occurs at 30 min post-injection, but also that the protein is truncated (Fig. 3). As early as 5 min (Fig. 3b) after i.p. injection, a peak at 7620 Da corresponding to [$^{44}\text{AANA}^{47}$]-RANTES was visible in serum, as well as a peak at 7436 Da corresponding to the predicted mass of [$^{44}\text{AANA}^{47}$]-RANTES lacking the N-terminal Ser¹–Pro² dipeptide (7439.5 Da). The rapid truncation of [$^{44}\text{AANA}^{47}$]-RANTES in serum correlates with previous observations that a significant ratio of RANTES isolated from hPBMC cell cultures is the truncated version missing the N-terminal dipeptide, designated 3–68 RANTES (Noso et al., 1996). The maximal concentration of [$^{44}\text{AANA}^{47}$]-RANTES in serum is attained 30 min (Fig. 3c) after i.p. injection consisting virtually only of the 3–68 truncated variant of the protein. 90 min (Fig. 3d) after injection the concentration of [$^{44}\text{AANA}^{47}$]-RANTES in serum had dropped significantly compared to 30 min and a new peak at 7273 Da was detected, corresponding to the theoretical mass of [$^{44}\text{AANA}^{47}$]-RANTES lacking the three N-terminal peptides Ser¹–Pro²–Tyr³ (7276.3 Da). This corresponds to the RANTES variant designated 4–68 (Lim et al., 2005). The overall concentration of the protein had further dropped after 4 h (Fig. 3e), however small peaks corresponding to both the 3–68 and the 4–68 variant of the protein could still be detected. After 6 h (Fig. 3f), [$^{44}\text{AANA}^{47}$]-RANTES levels had dropped below the detection limit of the method used.

A drawback of this technology is the variation between the spots due to the handling of very small volumes on different ProteinChip Arrays or even within the same chip (Supplementary table). A further source of imprecision could arise from possible manufacturing differences between different ProteinChip Array lots as well as small differences in laser intensities and

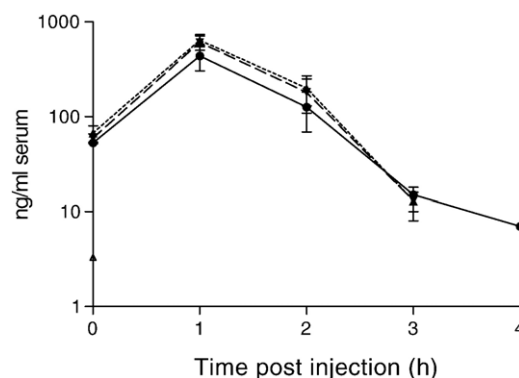


Fig. 5. Pharmacokinetic values of human [$^{44}\text{AANA}^{47}$]-RANTES in mouse serum as measured by ELISA and SELDI. ELISA (filled diamonds), SELDI peak height (filled squares) and SELDI peak area (filled circles). Each data point represents the average value obtained for three mice with measurements in triplicate.

alignment on the different spots on a chip. The variations (for details see Supplementary table) can of course also be attributed to the variations inherent in studies with animals, and will be reduced by increasing the group size if higher precision is required. In this evaluation of the technology we used triplicate measurements of each condition and three mice per time point in order to measure the PK value. The supplementary table represents all the single measures and Fig. 4 represents [$^{44}\text{AANA}^{47}$]-RANTES spectra of three different mice per time point. These data show that there are evidently some variations between the triplicates of one condition as well as between different mice, but when averaging the data the variations seem to be accounted for.

In summary the PK values obtained with SELDI correlate well with those obtained by ELISA for [$^{44}\text{AANA}^{47}$]-RANTES (Fig. 5). Furthermore, the oxidation of the protein observed after incubation in serum to construct the standard curve is also observed in the *in vivo* samples. Each peak analyzed from these samples is present as a doublet with a difference of 16 Da for each truncated form. Details of the degradation of the protein are seen on the spectra. Thus this method of PK determination provides important information about the protein of interest in *in vivo* conditions, giving it an added advantage over the use of ELISA for PK studies.

4. Discussion

Analysis of small molecule drugs and their metabolites in biological fluids is routinely performed by well established technologies such as LC-MS. However the analysis of therapeutic proteins is limited to measurements by ELISA or measurements of surrogate markers

to follow their fate *in vivo*. The recently developed SELDI technology which combines specific capture by an antibody followed by quantitative mass spectrometry provides a possibility for the development of a method for the bio-analysis of certain therapeutic proteins in biological fluids.

We have applied this technology to investigate preliminary pharmacokinetic studies and have shown that it produced a similar pattern of half-life and bio-distribution as those determined by ELISA, whilst demonstrating several additional advantages. Early PK data are obtained sooner with this technology as compared to ELISA since polyclonal antibodies can be used which are usually available within two to three months compared to the production of monoclonal antibodies which can take up to nine months. The results obtained here demonstrate that the limits of detection are similar with the two methods for this test protein. Both methods can detect protein down to 10 ng/ml concentrations. Errors occurring from antibody cross-reactivity are excluded when using the SELDI technology since the data are obtained precisely from the molecular entity of interest. Furthermore, in the absence of antibodies that discriminate between different protein isoforms, SELDI-TOF mass spectrometry remains the only possible tool for accurate sample analysis (Buhimschi et al., 2005).

SELDI has been used successfully for a variety of applications, including differential protein expression mapping to identify disease state related biomarkers (Reddy and Dalmasso, 2003), protein–protein/small molecule interaction studies (Amaar et al., 2002), monitoring enzymatic reactions (Boyle et al., 2001), identification of pathways specific to disease (Moscova et al., 2006) and detection of protein isoforms (Rossi et al., 1997) and protein quantification in biological samples. For differential protein expression mapping the MS spectra from various biological conditions are compared and the relative expression levels of proteins at specific molecular weights are determined by various statistical techniques and bioinformatic software systems. This is today an unmatched tool for multimarker clinical diagnostics, both for evaluating the disease state in serum and tissue and for analyzing drug responder groups, and has shown increased sensitivity compared to formerly used monomarker diagnostic assays in prostate, breast and ovarian cancer (Reddy and Dalmasso, 2003).

An additional advantage of the SELDI technology is that early processing events can be monitored. Chemical modifications such as oxidation pose a problem in the production of protein therapeutics, and considerable efforts are taken in the purification and formulation of proteins to prevent such modifications (Krishnamurthy

and Manning, 2002). The results shown here demonstrate that this modification occurs on exposure to serum, despite the fact that this RANTES protein has not undergone oxidation after storage for more than three years as a lyophilized powder. However, not all chemical modifications can be monitored by mass measurements performed on this type of instrument, as an example the resolution of the SELDI mass analyzer would be too low for the detection of deamidation (+1 Da).

The method may also have limitations for high molecular weight or highly glycosylated proteins that are not always well detected by mass spectrometry. Glycosylated proteins appear as broad peaks on MALDI-TOF MS spectra. Small mass differences of 16 Da (oxidation) within these proteins will be difficult to detect. However, the method could be applied to recombinant proteins produced in CHO cells, especially when the protein has the identical sequence to the human protein resulting in the difficulty of obtaining an ELISA capable of differentiating the protein that is administered to man from the endogenous protein. The glycosylation pattern of proteins produced in mammalian recombinant cell expression systems differs extensively which would thus allow the identification of the protein in serum samples.

One of the major advantages of the SELDI technology is the ability to follow enzymatic modifications that occur *in vivo*. The most widely studied example of natural chemokine truncation is through dipeptidylpeptidase IV (DPP IV) also known as CD26 (van Damme et al., 1999). CD26 catalyzes the removal of N-terminal dipeptides from proteins where the penultimate amino acid is either a proline or an alanine. A truncated version of RANTES, [3–68]-RANTES, is generated in the presence of CD26 resulting in a protein with a significantly altered biology. [3–68]-RANTES is a selective ligand for CCR5 (Iwata et al., 1999; Struyf et al., 1998) with a loss of chemotactic potency for monocytes and eosinophils (Proost et al., 1998; Struyf et al., 1999). In the chemokine protein family, truncation of the amino terminal region has also been described resulting from cleavage by matrix metalloproteinases (MMPs) and chymase *in vitro* (Overall et al., 2002; Sadir et al., 2004). The formation of the 3–38 and 4–68 truncations of RANTES have previously been observed in human serum, and this processing pattern was confirmed here by the administration of the RANTES variant lacking GAG binding properties to mice. This technology will therefore be useful in confirming the truncation patterns described *in vitro* in biological samples, especially in diseased conditions where both enzymes such as MMPs and pro-inflammatory immune modulators are up-regulated. It has recently been

demonstrated that chemokine truncation *in vivo* may be particularly relevant in disease processes. Chemokines such as CCL15 and CCL23 have been shown to be weak CCR1 agonists *in vitro* yet become extremely potent following N terminal truncation (Berahovich et al., 2005). That these chemokines are detected at relatively high levels in synovial fluid from arthritis patients suggests that chemokines that have an important role in induction or maintenance of autoimmune disease following *in vivo* processing may have been previously overlooked.

To resume, the SELDI technology is an innovative tool for PK studies of protein therapeutics. Compared to the traditionally used ELISA, it provides nearly identical PK values. We obtained identical results measuring either peak area or peak height in the SELDI spectra. Variations due to experimental parameters were minimized by averaging values from measurements performed in triplicate and by randomizing the sample positions on the ProteinChip arrays. Precise calibration of the ProteinChip reader followed by rigorous alignment of the spectra over the mass range of interest and normalization of peak intensities using a selected reference peak (*R* in Figs. 2, 3 and 4) from serum are essential for the determination of reliable PK values. In addition using the SELDI technology we were able to observe rapid *in vivo* processing of the protein into an oxidized form and successively into two truncated variants lacking the first two or three N-terminal residues. This provides a clear advantage to the SELDI methodology compared to ELISA, at least when the therapeutic protein of interest is relatively homogeneous, and not heavily glycosylated.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jim.2006.10.001.

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