Extracellular matrix-induced signaling pathways and pancreatic beta cell survival

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THESE

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CONTENTS

ABBREVIATIONS USED IN THE TEXT

DD: Death domain

ECM : Extracellular matrix

ERK: Extra-cellular regulated kinase

FAK: Focal adhesion kinase

FasL: Fas ligand

GLP-1: Glucagon-like peptide 1

IAP: Inhibitor of apoptosis protein

IFN-γ: Interferon γ

IGF-I: Insulin-like growth factor I

IκB: Inhibitor of NF-κB

IKK: IκB kinase

IL-1β: Interleukin-1 beta

IMD: Integrin-mediated death

iNOS: inducible nitric oxide synthase

MAP kinase: Mitogen activated protein kinase

NF-κB: Nuclear factor κB

NO: Nitric oxide

PDGF: Platelet-derived growth factor

PDTC: Pyrrolidine dithiocarbamate

$PI(4,5)P_2$: Phosphatidyl inositol-4,5-biphosphate

PI(3,4,5,)P3: Phosphatidyl inositol-3,4,5-triphosphate

PI3K: Phosphatidylinositol 3-kinase

PKB/Akt: Protein kinase B

pLL: poly-L-lysine

SH2 domain: Src-homology 2 domain

TNFα: Tumor necrosis factor α

RESUME EN FRANÇAIS

1. Introduction

1.1. L'apoptose de la cellule bêta et le diabète

L'îlot pancréatique de Langerhans est composé de quatre différents types de cellules qui sécrètent des hormones biologiquement actives dans la circulation sanguine : les cellules alpha, bêta, delta et PP. La fonction majeure de ces cellules est de maintenir une concentration plasmatique de glucose (glycémie) dans une fourchette restreinte (3.5 à 7 mmol/l). L'augmentation de cette dernière, suite à un apport exogène de glucose (lors d'un repas), induit la sécrétion d'insuline par la cellule bêta pancréatique. L'insuline sécrétée induit alors une diminution de la glycémie, maintenant ainsi l'homéostasie glucidique. L'absence de régulation correcte de la glycémie est l'un des symptômes principaux indiquant la présence de diabète. Il existe deux types de diabète : le diabète de type 1 résultant d'une destruction auto-immune progressive des cellules bêta pancréatiques, et le diabète de type 2 caractérisé par une résistance à l'insuline des tissus cibles (tels que le foie et le muscle) accompagnée d'un défaut de sécrétion d'insuline par la cellule bêta. Cette sécrétion insuffisante d'insuline résulterait d'une combinaison d'un dysfonctionnement cellulaire inhérent d'une part, et d'une diminution de la masse de cellules bêta d'autre part. Récemment, la mort cellulaire par apoptose a été impliquée dans la diminution de la masse de cellules sécrétrices d'insuline non seulement dans le cas du diabète de type 1 mais aussi dans le cas du diabète de type 2. La cellule bêta semble être particulièrement sensible aux stimuli pro-apoptotiques, probablement à cause de son phénotype particulièrement bien différencié. Quels sont les facteurs induisant l'apoptose des cellules bêta ? Les principaux stimuli pro-apoptotiques supposés être impliqués dans la pathogénèse du diabète de type 2 comprennent les cytokines, la lipotoxicité, et la glucotoxicité.

Un modèle général pour expliquer le rôle de l'apoptose dans le développement du diabète de type 2 a été proposé. Au début de la maladie, au stade de la résistance à l'insuline, une augmentation de la masse des cellules bêta compensant la résistance à l'insuline peut être observée. Cependant, celle-ci est temporaire, et est suivie par un déclin progressif de la masse des cellules bêta, impliquant la mort cellulaire par apoptose. Le déclenchement du diabète proprement dit est caractérisé par une incapacité des cellules bêta résiduelles à compenser la résistance à l'insuline. Par conséquent, un état d'hyper-glycémie permanent et donc diabétique s'installe. La maintenance de la survie des cellules bêta et d'une masse de cellules bêta adéquate sont donc des stratégies prometteuses qui pourraient permettre soit de prévenir, soit de traiter le diabète de type 2. Toutefois, les mécanismes impliqués dans la protection des cellules bêta contre l'apoptose sont encore mal définis. Parmi ceux-ci, un élément encore mal connu pourrait contribuer à maintenir la survie des cellules bêta dans un environnement hostile : la matrice extracellulaire.

1.2. La matrice extracellulaire

1.2.1. Généralités

La matrice extracellulaire a été longtemps considérée uniquement comme un échafaudage inerte qui sert à maintenir la structure physique des tissus. Des recherches effectuées ces vingt dernières années ont permis de mettre en évidence que la matrice extracellulaire est une structure extrêmement dynamique, capable d'activer des voies de signalisation intracellulaires influençant un large spectre de fonctions biologiques telles que l'adhésion cellulaire, la migration, la prolifération, la différenciation et la survie des cellules. C'est en particulier le rôle anti-apoptotique de la matrice extracellulaire qui nous intéresse ici. En effet, il existe un type d'apoptose spécifique, appelé anoikis, qui est induit soit par le détachement des cellules de leur matrice extracellulaire, soit par l'interaction des cellules avec une matrice extracellulaire non-adaptée. L'apoptose peut être inhibée par différents facteurs « pro-survie », capables de contrebalancer les stimuli pro-apoptotiques. Parmi ces facteurs bénéfiques figure la matrice extracellulaire. Les mécanismes impliqués dans l'anoikis ne sont pas encore bien caractérisés. Néanmoins, il semble que l'activation de la caspase-8 soit souvent impliquée dans cette forme d'apoptose.

L'adhésion des cellules à la matrice extracellulaire s'effectue essentiellement par le biais d'une famille de récepteurs transmembranaires hétérodimériques : les intégrines. Ces dernières sont composées d'une sous-unité α et d'une sous-unité β. La famille des intégrines des vertébrés contient au moins 18 sous-unités α et 8 sous-unités β qui peuvent s'associer et former au moins 20 hétérodimères distincts. C'est la combinaison spécifique d'une sous-unité α avec une sous-unité β qui détermine l'affinité de l'hétérodimère pour une protéine spécifique de la matrice extracellulaire. Les ligands courants des intégrines sont des composants de cette matrice tels que le collagène, la fibronectine, la vitronectine, ainsi que la laminine. L'interaction d'une cellule avec une matrice extracellulaire induit un regroupement d'intégrines qui initie non seulement l'adhésion et l'organisation du cytosquelette via l'interaction physique des intégrines avec d'autres groupes de protéines, mais également l'activation de plusieurs voies de signalisation intracellulaires. Ces voies de signalisation intracellulaires ont été impliquées dans l'effet anti-apoptotique de la matrice extracellulaire.

1.2.2. *Voies de signalisation induites par la matrice extracellulaire*

L'engagement des intégrines à une matrice extracellulaire adaptée peut induire l'activation d'un grand nombre de protéines impliquées dans des voies de signalisation intracellulaires. L'effet anti-apoptotique de la matrice extracellulaire peut être transmis par différentes cascades de signalisation impliquant les protéines Focal Adhesion kinase (FAK), Phosphatidylinositol 3-kinase (PI3K), protéine kinase B (PKB/Akt), les MAP kinases (MAPK) ERK1 et ERK2 (ERK1/2), ainsi que par l'activation du facteur de transcription NF-κB.

FAK, une protéine localisée aux sites d'adhésion de la cellule avec la matrice extracellulaire (les adhésions focales), est une des premières protéines découverte pouvant être activée suite à l'interaction des intégrines avec leur ligand extracellulaire. Cette interaction induit la phosphorylation de la FAK sur des résidus tyrosine (le résidu tyrosine-397, notamment), ce qui conduit au recrutement de protéines adaptatrices et signalisatrices par la FAK. La phosphorylation du résidu tyrosine-397 est à l'origine du recrutement de la PI3K par FAK. De plus, l'activation de la FAK peut également induire l'activation de la voie des MAPK ERK1/2. Par conséquent, la phosphorylation du résidu tyrosine-397 de la FAK est souvent considérée comme un marqueur indiquant la présence d'intégrines activées.

Une fois activée, la PI3K peut à son tour stimuler plusieurs protéines signalisatrices. Un des effecteurs majeurs de la PI3K est PKB/Akt (également appelée Akt). La phosphorylation d'Akt sur les résidus thréonine-308 et sérine-473 corrèle avec son niveau d'activation : la phosphorylation de ces résidus est donc considérée comme un marqueur indiquant l'activation de la voie PI3K-Akt. En général, la voie PI3K-Akt est considérée comme étant une voie de signalisation intracellulaire anti-apoptotique majeure.

La voie des MAPK ERK1/2 a été impliquée dans la régulation de plusieurs fonctions cellulaires majeures telles que la migration cellulaire, la prolifération et la survie des cellules. Il existe deux isoformes de la protéine ERK : ERK1 et ERK2 (également appelés p44 et p42, respectivement). L'activation des ERK1/2 est induite par une double phosphorylation sur deux résidus (thréonine et tyrosine). Une fois phosphorylés, les ERK1/2 peuvent à leur tour activer un nombre important d'effecteurs, incluant des protéines membranaires et cytoplasmiques, ainsi que des facteurs de transcription.

Deux mécanismes principaux ont été impliqués dans l'effet anti-apoptotique des voies de signalisation induites par la matrice extracellulaire et transmises par FAK, PI3K-Akt et ERK1/2. Le premier induirait un effet direct sur la phosphorylation et donc sur l'activité des protéines impliquées dans l'apoptose. Le second mécanisme aurait lieu via une altération du niveau de l'expression de gènes impliqués dans la régulation de la survie cellulaire (à travers une régulation de l'activité des facteurs de transcription). Durant ce travail de thèse, nous nous sommes particulièrement intéressés à ce dernier mécanisme. En particulier, nous nous

sommes penchés sur l'effet de la matrice extracellulaire sur l'activité du facteur de transcription NF-κB.

La famille des facteurs de transcription NF-κB est composée de cinq membres : p65 (RelA), RelB, c-Rel, p50/p105 (NF-κB1) et p52/p100 (NF-κB2). Celui sur lequel nous nous sommes focalisés est l'hétérodimère communément appelé « NF-κB », composé des protéines p65 et p50. NF-κB a été impliqué dans la régulation de nombreuses fonctions biologiques et cellulaires, telles que l'inflammation, la prolifération, la migration et la survie des cellules. Dans une cellule non-stimulée, NF-κB est séquestré dans le cytoplasme par des protéines appelées « inhibitor of NF-κB », les IκBs. L'activation de la cellule par un stimulus donné (tel que la cytokine IL-1β ou la matrice extracellulaire) semble induire la phosphorylation et l'activation d'un groupe de kinases : les IκB kinases (IKK), qui phosphorylent les IκBs. La phosphorylation des IκBs entraîne leur ubiquitination et par conséquent leur dégradation. Il en résulte la libération de NF-κB menant à sa translocation vers le noyau. Une fois dans le noyau, NF-κB va se lier à des sites promoteurs particuliers et va ainsi induire l'expression de gènes spécifiques (tels que IκBα, le chemokine IP-10 et le facteur de transcription c-Myc). IκBα est un des gènes-cible majeurs de NF-κB, et ceci constitue un mécanisme rétro-actif inhibant l'activité transcriptionnelle de NF-κB. L'activité transcriptionnelle de NF-κB est régulée non seulement via la dégradation d'IκB, mais aussi par la phosphorylation et/ou l'acétylation directe de la sous-unité p65, ce qui module le potentiel de transactivation de NF-κB. Plusieurs protéines et voies de signalisation ont été impliquées dans l'activation de NF-κB, telles que la protéine FAK et les cascades de signalisation impliquant PI3K-Akt et ERK1/2.

Il a été démontré que NF-κB peut jouer un rôle pro-apoptotique ou anti-apoptotique suivant le contexte, la nature, l'intensité et la durée du stimulus induisant son activité, ainsi que le type cellulaire étudié. L'activation de NF-κB par la matrice extracellulaire a été impliquée dans l'effet anti-apoptotique de cette dernière. De plus, les kinases FAK, PI3K, Akt et ERK1/2 ont toutes été impliquées dans le rôle anti-apoptotique de NF-κB.

En résumé, la matrice extracellulaire peut activer les kinases FAK, PI3K, Akt et ERK1/2, ainsi que le facteur de transcription NF-κB. De plus, un rôle anti-apoptotique a été proposé pour toutes ces protéines, et toutes ont été impliquées dans l'effet pro-survie de la matrice extracellulaire. Néanmoins, les rôles éventuels de ces protéines dans les effets de la matrice extracellulaire sur la cellule bêta pancréatique étaient inconnus au début de ce travail de thèse.

1.3. La matrice extracellulaire et la cellule bêta pancréatique

Avant le début de ce travail de thèse, quelques travaux rapportant que la matrice extracellulaire pourrait améliorer la survie ainsi que la fonction (sécrétion d'insuline stimulée par le glucose) des cellules bêta en culture avaient été publiés. Etant donné que la laminine et le collagène IV sont présents dans la membrane basale des îlots de Langerhans, notre groupe s'est intéressé à l'expression des intégrines dans les cellules bêta de rat. Il a été observé que ces dernières expriment les intégrines $\alpha 6\beta 1$ et $\alpha 3\beta 1$, qui sont des récepteurs de la laminine-5. D'autre part, notre groupe a découvert qu'une matrice extracellulaire riche en laminine-5 (appelée 804G-ECM) induit l'étalement des cellules bêta de rat et améliore leur sécrétion d'insuline en réponse au glucose. Les interactions entre la laminine-5 et l'intégrine $\alpha 6\beta 1$ semblent être responsables, du moins en partie, des effets bénéfiques de la matrice 804G-ECM sur la cellule bêta.

Les voies de signalisation induites par la matrice extracellulaire en général ou par la matrice 804G-ECM en particulier dans la cellule bêta étaient complètement inconnues lors du début de ce travail de thèse. Aucun travail décrivant l'expression ou la fonction de FAK dans la cellule bêta n'avait alors été publié. Néanmoins, plusieurs papiers suggérant que les voies de signalisation PI3K-Akt et MAPK ERK1/2 puissent jouer un rôle dans le contrôle de la survie de la cellule bêta ont été publiés soit avant, soit pendant ce travail de thèse. La voie de signalisation PI3K-Akt est en général considérée comme une voie anti-apoptotique dans la cellule bêta, mais cela n'est pas encore complètement établi. Quant à la voie des MAPK ERK1/2, son éventuel rôle anti-apoptotique dans la cellule bêta est encore controversé.

La fonction du facteur de transcription NF-κB dans la cellule bêta dans le contexte de la mort cellulaire induite par les cytokines (IL-1β, en particulier) a par contre été abondamment documentée. En effet, plusieurs travaux ont démontré que la cytokine IL-1β active NF-κB et que NF-κB est impliqué dans les effets néfastes de cette cytokine menant à un dysfonctionnement et à la mort de la cellule bêta. NF-κB est donc considéré comme une molécule transmettant des signaux pro-apoptotiques dans la cellule bêta, dans le contexte du diabète de type 1 et de la mort cellulaire induite par les cytokines. Néanmoins, d'autres travaux suggèrent que NF-κB, activé par des stimuli autres que les cytokines, pourrait avoir un rôle qui diffère des effets délétères sus-mentionnés. Notamment, il semble que NF-κB puisse être impliqué dans l'effet anti-apoptotique de GLP-1 (« Glucagon-like peptide 1 ») sur les cellules bêta.

2. But général du projet de thèse

Le but général de ce travail de thèse était de répondre aux questions suivantes :

- Est-ce que la matrice 804G-ECM protège les cellules bêta contre l'apoptose ?

- Est-ce que la matrice 804G-ECM active les kinases FAK, PI3K-Akt et ERK1/2 dans la cellule bêta ; si oui, est-ce que ces protéines sont impliquées dans l'effet pro-survie de la matrice 804G-ECM ?

- Est-ce que la matrice 804G-ECM est capable d'induire l'expression de gènes, et est-ce que cette régulation est impliquée dans ses effets sur la cellule bêta ?

Ce travail de thèse est composé de deux articles qui ont été publiés dans des revues internationales. Le but du premier article était d'analyser l'effet de la matrice 804G-ECM sur la survie des cellules bêta ainsi que son impact sur l'activité des kinases FAK, PI3K-Akt et ERK1/2. L'implication des voies de signalisation PI3K-Akt et ERK1/2 dans l'effet pro-survie de la matrice 804G-ECM a été évaluée en utilisant des inhibiteurs pharmacologiques. D'autre part, nous avons déterminé si la matrice 804G-ECM peut modifier l'expression de gènes dans la cellule bêta. Durant cette première partie de la thèse, nous avons notamment découvert que la matrice 804G-ECM induit l'expression de l'inhibiteur de NF-κB : IκBα. IκBα est un gène-cible de NF-κB, suggérant que la matrice 804G-ECM pourrait avoir un impact sur l'activité transcriptionnelle de NF-κB. Le deuxième papier est focalisé sur l'activation de NF-κB induite par la matrice 804G-ECM et sur son rôle dans les effets de cette dernière sur la cellule bêta : l'étalement, l'organisation du cytosquelette, la fonction (sécrétion d'insuline en réponse au glucose), ainsi que la survie. Etant donné que NF-κB est en général considéré comme une molécule transmettant les effets néfastes des cytokines tels qu'IL-1β sur la cellule bêta, la possibilité qu'il puisse être impliqué dans les effets *positifs* de la matrice 804G-ECM sur la cellule bêta était fascinante. De plus, l'implication des voies de signalisation PI3K-Akt et MAPK ERK1/2 dans l'activation de NF-κB induite par la matrice 804G-ECM est également abordée dans cet article.

Le niveau d'expression des intégrines, l'effet de la matrice extracellulaire et de ses différents composants sur la signalisation intracellulaire et sur la survie des cellules diffèrent suivant les types cellulaires étudiés. De plus, ces caractéristiques diffèrent également entre les cellules primaires et les lignées cellulaires (transformées ou pas) qui en dérivent. Par conséquent, nous avons décidé d'étudier exclusivement les cellules bêta primaires de rat purifiées par FACS.

3. Résumé des travaux

Article 1: La matrice extracellulaire protège les cellules bêta pancréatiques de l'apoptose : rôles des voies de signalisation à court et à long terme

" Extracellular Matrix Protects Pancreatic Beta-Cells Against Apoptosis : Role of Short- and Long-Term Signaling Pathways ", Hammar et al. Diabetes, 53: 2034-2041 (2004)

La matrice 804G-ECM a été utilisée comme modèle afin de déterminer si une matrice extracellulaire adaptée peut protéger les cellules bêta pancréatiques de la mort cellulaire. L'effet de cette matrice sur la survie des cellules bêta a été testée après 48h de culture, dans trois différentes conditions : en présence d'une concentration standard de sérum (10% FCS), en présence d'une basse concentration de sérum (1% FCS), ainsi qu'en présence de la cytokine IL-1β. Les cellules contrôle étaient cultivées soit sur des pétris non recouverts de matrice, soit sur des pétris recouverts d'un substrat adhésif « neutre » : la poly-L-lysine (pLL). Nous avons observé que la matrice 804G-ECM protège les cellules de l'apoptose et de la nécrose, dans toutes les conditions testées. Comme l'activation de la caspase-8 est souvent impliquée dans l'anoikis, nous avons mesuré cette activité dans les cellules cultivées sur la pLL et sur la matrice 804G-ECM. L'activité de la caspase-8 est significativement diminuée dans les cellules cultivées sur la matrice 804G-ECM par rapport aux cellules contrôle. Ces résultats impliquent que la matrice 804G-ECM protège les cellules bêta de l'apoptose et suggèrent que la caspase-8 pourrait être impliquée dans la mort de ces cellules cultivées sur la pLL.

La matrice 804G-ECM est surtout composée de laminine-5, mais aussi d'autres protéines, telle que la fibronectine. C'est pourquoi nous avons vérifié que son effet anti-apoptotique est bien une conséquence de l'engagement de la laminine-5 à ses récepteurs, les intégrines α3β1 et α6β1. Nous avons d'abord testé l'effet de la laminine-5 purifiée sur l'étalement et la survie des cellules, et nous avons constaté que son effet est similaire à celui de la matrice 804G-ECM. L'effet d'un anticorps bloquant l'intégrine β1 sur la survie des cellules bêta cultivées sur pLL et sur la matrice 804G-ECM a également été évalué. Le blocage de l'intégrine β1 induit une inhibition partielle mais significative de l'effet anti-apoptotique de la matrice 804G-ECM. Ceci implique que les interactions entre la laminine-5 et l'intégrine β1 sont responsables, du moins en partie, de l'effet anti-apoptotique de la matrice 804G-ECM sur les cellules bêta.

Chacune des protéines FAK, Akt et ERK1/2 est susceptible de transmettre le signal anti-apoptotique généré par l'interaction des intégrines avec une matrice extracellulaire adaptée. C'est pourquoi nous avons étudié l'effet de la matrice 804G-ECM sur l'activité de ces kinases dans la cellule bêta. Nous avons entrepris des transfert Western (Western blotting) afin d'évaluer si la matrice 804G-ECM induit une phosphorylation rapide (30 minutes) de FAK (sur le résidu tyrosine-397), de Akt (sur résidu sérine-473) et de ERK1/2 (sur les résidus thréonine-202 et tyrosine-204). Nous avons constaté que le niveau de phosphorylation de ces protéines sur

ces résidus est plus élevé dans les cellules cultivées sur la matrice 804G-ECM par rapport aux cellules cultivées sur la pLL. Dans un deuxième temps, l'effet de l'inhibition des voies de signalisation impliquant Akt et ERK1/2 a été étudié en utilisant des inhibiteurs pharmacologiques. Les résultats obtenus montrent que les inhibiteurs de PI3K (LY294002) et de MEK1 (PD98059) diminuent de manière partielle mais significative l'effet anti-apoptotique de la matrice 804G-ECM après 4h de culture, alors que la survie des cellules contrôle n'était pas altérée par ces inhibiteurs. Lors des études à plus long terme (48h), l'inhibiteur de PI3K n'a plus d'effet significatif sur la survie des cellules bêta, qu'elles soient cultivées sur la pLL ou la matrice 804G-ECM. En revanche, l'inhibiteur de la voie ERK1/2 supprime de manière drastique l'effet anti-apoptotique de la matrice 804G-ECM, et augmente également la mortalité des cellules contrôle. Ceci implique que la voie des MAPK ERK1/2 joue un rôle majeur dans l'effet anti-apoptotique à long terme de la matrice 804G-ECM.

Afin d'étudier la possibilité que la matrice 804G-ECM induise l'expression de gènes qui pourraient être impliqués dans son effet anti-apoptotique, un « microarray » à basse densité suivi par une validation par RT-PCR quantitative ont été effectués. L'ARNm extrait de cellules bêta cultivées pendant 18h en présence ou en absence de la matrice 804G-ECM a été utilisé pour cette expérience. Les résultats obtenus ont montré que la matrice 804G-ECM induit une surexpression de l'inhibiteur de NF-κB (IκBα). Afin de déterminer si les voies PI3K-Akt ou MAPK ERK1/2 sont impliquées dans ce phénomène, l'effet de l'inhibition de ces voies sur la surexpression d'IκBα induite par la matrice 804G-ECM a été analysé. Nous avons constaté que l'inhibition de l'activité d'ERK1/2 diminuait de manière partielle la surexpression d'IκBα induite par la matrice 804G-ECM, tandis que la suppression de l'activité de PI3K n'avait pas d'effet. Ces résultats suggèrent que la voie ERK1/2 est en partie impliquée dans la surexpression d'IκBα induite par la matrice 804G-ECM. Deux hypothèses principales ont été émises suite à ces résultats. D'abord, comme IκBα est un gène-cible du facteur de transcription NF-κB, nous avons supposé que la matrice 804G-ECM pourrait induire l'activité transcriptionnelle de NF-κB. Ensuite, nous avons évoqué la possibilité que NF-κB pourrait être impliqué dans les effets de la matrice 804G-ECM sur la cellule bêta pancréatique ; à savoir l'étalement, l'organisation du cytosquelette, la sécrétion d'insuline en réponse au glucose et la survie cellulaire. Ces hypothèses constituent les bases de la deuxième partie de la thèse et de l'article 2.

<u>Article 2:</u> L'activation de NF-κB par la matrice extracellulaire est impliquée dans l'étalement et la sécrétion d'insuline stimulée par le glucose des cellules bêta pancréatiques

"Activation of NF-κB by Extracellular Matrix Is Involved in Spreading and Glucose-stimulated Insulin Secretion of Pancreatic Beta Cells", Hammar et al. J Biol Chem, 280: 30360-30637 (2005)

Le but de ce deuxième article était d'établir si la matrice extracellulaire induit une activité transcriptionnelle de NF-κB et d'étudier si l'activité de ce facteur de transcription est impliquée dans un ou plusieurs effets de la matrice 804G-ECM sur la cellule bêta de rat. Nous avons observé qu'une heure d'exposition des cellules bêta à la matrice 804G-ECM induit une augmentation significative de la translocation nucléaire et de l'activité de liaison à l'ADN de la sous-unité p65 de NF-κB (appelée NF-κB par la suite) par rapport au substrat contrôle pLL. L'activité de liaison à l'ADN de NF-κB induite par la matrice 804G-ECM commence à augmenter après 30 minutes de culture, est maximale entre 1h-2h, et diminue après 4h de culture. L'expression de deux gènes-cible de NF-κB (IκBα et NF-κB1 (p105)) induite par la matrice 804G-ECM en fonction du temps a été mesurée par RT-PCR quantitative. La surexpression de ces deux gènes est maximale après 4h d'exposition à la matrice 804G-ECM. Cette surexpression semble être transitoire, car elle disparaît après 24h de culture. Le schéma temporel de l'expression des deux gènes-cible de NF-κB induite par la matrice 804G-ECM corrèle donc avec celui de l'augmentation de son activité transcriptionnelle induite par cette matrice extracellulaire. La surexpression de la protéine IκBα induite par la matrice 804G-ECM a été confirmée par transfert Western (Western blotting). De plus, nous avons observé que la translocation nucléaire et l'activité de liaison à l'ADN de NF-κB induites par la cytokine IL-1β (2ng/ml, 20 minutes) sont nettement plus intenses que celles induites par la matrice 804G-ECM. Ces résultats impliquent que la matrice 804-ECM induit une activité transcriptionnelle transitoire et modérée (par rapport à la cytokine IL-1β) de NF-κB.

Afin d'étudier les effets de l'activité de NF-κB induite par la matrice 804G-ECM sur la cellule bêta, deux approches différentes ont été utilisées : d'une part, un inhibiteur pharmacologique empêchant la phosphorylation et la dégradation d'IκBα (Bay 11-7082) et d'autre part un adénovirus surexprimant IκBα sous la forme d'une protéine mutante non-dégradable. Cette dernière approche n'a pas été utilisée pour déterminer l'effet de l'inhibition de l'activité de NF-κB sur la sécrétion d'insuline, car nous avons constaté que le virus contrôle inhibe de façon non-spécifique la sécrétion d'insuline induite par le glucose dans notre système (cellules bêta de rat triées par FACS). Contrairement à notre hypothèse de base, l'inhibition de l'activité de NF-κB par les deux approches sus-mentionnées n'a aucun effet sur la survie des cellules, quelque soit le substrat de culture (le contrôle pLL ou la matrice 804G-ECM). En revanche, l'inhibition de NF-κB réduit de manière significative l'étalement des cellules et

empêche l'organisation du cytosquelette d'actine induite par la matrice 804G-ECM. Finalement, l'inhibiteur pharmacologique Bay 11-7082 réduit de manière dose-dépendante la sécrétion d'insuline stimulée par le glucose, suggérant qu'une activité modérée de NF-κB est indispensable à la sécrétion d'insuline. Du fait que NF-κB est impliqué dans les effets de la matrice 804G-ECM sur le cytosquelette d'actine et sur l'expression de gènes (IκBα), notre hypothèse pour les futures expériences est que ces deux paramètres sont impliqués dans le rôle de NF-κB dans la sécrétion d'insuline.

Comme la matrice 804G-ECM active les voies de signalisation PI3K-Akt et MAPK ERK1/2, leur rôle dans l'activation de NF-κB par la matrice 804G-ECM a été étudié. L'effet des inhibiteurs de PI3K (LY294002) et de MEK1 (PD98059) sur l'activité de liaison à l'ADN de NF-κB ainsi que sur la surexpression de l'ARNm d'IκBα induite par la matrice 804G-ECM a été analysé. Nous avons observé que l'inhibiteur de la voie d'ERK1/2 réduit l'activité de liaison à l'ADN de NF-κB et la surexpression d'IκBα induites par la matrice 804G-ECM, tandis que l'inhibiteur de PI3K n'affecte aucun de ces effets induits par cette matrice extracellulaire. La voie MAPK ERK1/2 pourrait donc jouer un rôle important dans l'activation de NF-κB par la matrice 804G-ECM. Néanmoins, il reste à éclaircir pourquoi l'inhibition de NF-κB et l'inhibition de la voie d'ERK1/2 n'ont pas les mêmes effets phénotypiques sur la cellule bêta. En effet, l'inhibition d'ERK1/2 inhibe l'effet pro-survie de la matrice 804G-ECM, tandis que l'inhibition de NF-kB n'affecte pas la survie de la cellule bêta. Nous proposons qu'un effecteur d'ERK1/2 autre que NF-κB pourrait être impliqué dans les effets de cette voie des MAPK sur la cellule bêta, menant à une divergence de ces deux voies de signalisation.

4. Conclusion

Nos résultats montrent que la matrice extracellulaire a un impact important sur la cellule bêta : elle améliore sa survie et elle induit l'activation des voies de signalisation impliquant les kinases FAK, PI3K, Akt et ERK1/2. De plus, elle active le facteur de transcription NF-κB, menant à la surexpression d'IκBα. Nous avons vu que les voies de signalisation intracellulaires PI3K-Akt et ERK1/2 sont les deux impliquées dans l'effet anti-apoptotique de la matrice 804G-ECM à court terme, tandis que seulement la voie ERK1/2 joue un rôle dans l'effet anti-apoptotique de cette matrice à long terme. De plus, ERK1/2 semble être impliquée dans l'activation de NF-κB induite par la matrice 804G-ECM. Contrairement à l'hypothèse émise au début de la deuxième partie de cette thèse, le facteur de transcription NF-κB ne semble pas être responsable de l'effet anti-apoptotique de la matrice 804G-ECM. Par contre, NF-κB semble être impliqué dans l'organisation du cytosquelette d'actine et l'étalement des cellules induits par la matrice 804G-ECM, ainsi que dans la sécrétion d'insuline stimulée par le glucose. Ce dernier résultat est renforcé par une étude récente (par Norlin et al.) effectuée sur des souris transgéniques surexprimant un mutant non-dégradable d'IκBα (qui inhibe NF-κB) spécifiquement dans les cellules bêta. Ces souris transgéniques ont une sécrétion d'insuline en réponse au glucose défectueuse et une expression des gènes impliqués dans l'exocytose de l'insuline perturbée. Ces travaux, en plus de nos résultats, suggèrent fortement qu'une activité modérée de NF-κB est indispensable à la fonction de la cellule bêta pancréatique. Il est intéressant de constater que le rôle *positif* de NF-κB dans la cellule bêta que nous proposons ici diffère considérablement du rôle délétère de NF-κB (dans le contexte d'une activation induite par des cytokines) rapporté par un certain nombre de travaux. Nous proposons que l'activité de NF-κB induite par la matrice 804G-ECM ou par les cytokines diffère aussi bien par son intensité que par sa durée. D'autre part, il est possible que la matrice 804G-ECM et les cytokines induisent une phosphorylation de la sous-unité p65 sur des sites différents, ce qui pourrait mener à des effets biologiques différents. Ces hypothèses constituent les bases de nos futurs travaux. En effet, il nous semble primordial de mieux comprendre les mécanismes impliqués dans la régulation de l'activité de NF-κB menant à des conséquences biologiques aussi contrastées, suivant le contexte de son activation. La prochaine étape serait d'inhiber spécifiquement certains aspects de son activité (menant à ses effets néfastes), sans pour autant affecter ses effets positifs sur la fonction de la cellule bêta pancréatique.

Finalement, nos résultats suggèrent que la matrice 804G-ECM induit une activité des kinases (FAK, PI3K, Akt et ERK1/2) et du facteur de transcription NF-κB indispensable au maintien de la fonction et de la survie de la cellule bêta pancréatique. Ainsi, la présence d'une matrice extracellulaire adéquate pourrait jouer un rôle important *in vivo* dans la protection des cellules bêta contre les effets délétères induits par des stimuli impliqués dans la pathogénèse du diabète.

PART I: GENERAL INTRODUCTION

1. Regulation of pancreatic beta cell mass and diabetes

1.1. Structure and function of the pancreatic islet of Langerhans

The pancreatic islet of Langerhans consists of four main cell types that secrete biologically active hormones into the circulation. The islets are composed of insulin-producing beta cells (60-80%) and of other endocrine cell types secreting glucagon (alpha cell, 25%), somatostatin (delta cell, 2-8%), and pancreatic polypeptide (PP cell, 1-2%). The distribution of these endocrine cells is non-random, with a core of beta cells, surrounded by non-beta cells. The islet of Langerhans is highly vascularized and innervated. In addition, endocrine cells are in contact with extracellular matrix (ECM) molecules contained in the surrounding basement membrane (1; 2), (Fig. 1).

One of the main functions of the islet hormones is to maintain a proper level of blood glucose. Insulin, which is secreted in response to high blood glucose, primarily functions to trigger glucose/energy storage for later use, by stimulating liver and muscle to synthesize glycogen and protein, and adipose cells to synthesize fat (triacylglycerols). Glucagon, which is secreted in response to low blood glucose, has essentially the opposite effect: it stimulates the release of glucose and of fatty acids. There is a complex interaction between the different islet cell types, which determines the secretory activity of the islet.

The principal characteristic of beta cells is that they function as “fuel-sensors” capable of adapting the rate of insulin secretion to the variations in plasma levels of glucose (the most important controller of insulin secretion) and other energetic substrates (amino acids, fatty acids, and ketone bodies). Furthermore, several hormones and neurotransmitters also exert control on beta cell function acting as potentiators or inhibitors of glucose-stimulated insulin secretion. Insulin is stored in vesicles and is released by exocytosis, a multistage process involving transport of vesicles to the plasma membrane, their docking, priming and finally their fusion with the plasma membrane (3).

As described above, beta cells form the core of the islet, a structure favoring intercellular contacts. Interestingly, it has been reported that homologous contacts between beta cells promote secretion of insulin by individual cells (4). This effect seems to be specific, as the heterologous contacts of beta cells with non-beta cells do not increase insulin secretion (4). Furthermore, as it will be discussed more in detail below, ECM is also able to increase secretion of individual pancreatic beta cells in response to glucose (5).

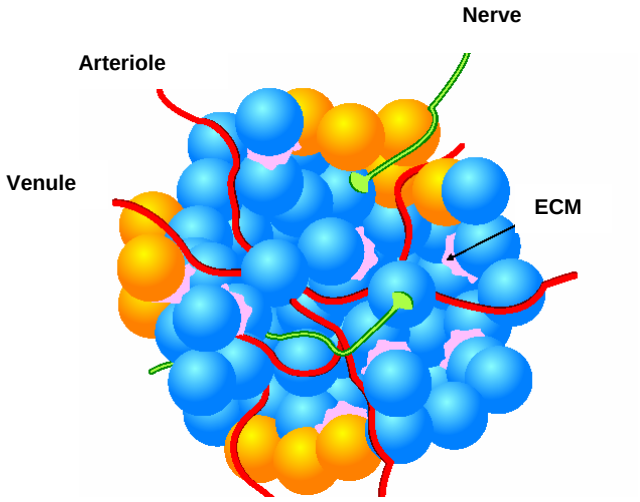

Fig.1: A pancreatic islet of Langerhans, with a core composed of beta cells (blue spheres), surrounded by non-beta cells (yellow spheres). The islet is richly vascularized and innervated, and the cells are in contact with an ECM that is presumably secreted by surrounding endothelial cells. Reproduced and adapted from reference (6).

1.2. The pancreatic beta cell and diabetes

1.2.1. Pancreatic beta cell mass and diabetes

The islet cell mass is dynamic, and is able to adjust to meet changing metabolic demands. The homeostatic control of beta cell mass in normal and pathological conditions is based on the balance of proliferation, differentiation/neogenesis, regulation of size and death of the insulin-secreting cells (7; 8). In particular, beta cell functional mass in the adult is plastic. Pregnancy and obesity are two typical situations that require dramatic adaptations (increase) in the beta cell mass to compensate for changing insulin demands. Failure of the beta cell mass to adapt to the changing needs is believed to contribute to the development of diabetes (7-9).

Diabetes is a chronic disease with severe secondary complications, reaching epidemic proportions (for type 2 diabetes) and for which there is yet no cure. The two major forms of diabetes mellitus are type 1 and type 2. Type 1 diabetes is characterized by autoimmune destruction of the insulin-producing pancreatic beta cells, which typically results in the near total loss of these cells (10). Type 2 diabetes is characterized by insulin resistance and defective insulin secretion (11). It has been debated whether this defective insulin secretion is due to reduced beta cell mass or to an intrinsic secretory defect or both. Increasing evidence indicates that decreased functional beta cell mass is the hallmark of both type 1 and type 2 diabetes, underlying insulin insufficiency in both conditions, albeit to different degrees (12). However, studies performed on pancreatectomized rats have shown that large pancreatectomies (at least 50%) are required to induce mild/moderate hyperglycemia and/or insulin secretory defects, while only almost complete pancreatectomies lead to overt diabetes with severe insulin deficiencies (reviewed by (13)). Therefore, the decreased beta cell mass cannot be the only cause leading to insufficient insulin secretion to maintain normoglycemia. The defects in insulin secretion that characterize type 2 diabetes are thus likely to be a result of the combination of both cellular dysfunction and a reduction in beta cell mass (8; 11).

The importance of a proper regulation of the beta cell mass to maintain normoglycemia and thus to avoid the onset of type 2 diabetes is quite a novel concept. Furthermore, the mechanisms involved in the control of beta cell mass are still poorly understood. Nevertheless, as discussed below, cell death by apoptosis is now generally accepted to be a major mechanism involved in the decrease of beta cell mass that contributes to the pathogenesis of diabetes.

1.2.2. Apoptosis and diabetes

Two distinct modes of cell death, apoptosis or necrosis, can be distinguished because of differences in morphologic, biochemical and molecular changes. Apoptosis is a well-characterized form of cell death that involves condensation and fragmentation of nuclei, internucleosomal DNA cleavage and randomization of the distribution of phosphatidyl serine (PS) between the inner and outer leaflets of the plasma membrane (14; 15). This form of physiological cell death differs from necrosis, in which cells swell and disintegrate in an unordered manner, eventually leading to the destruction of the cellular organelles and finally rupture of the plasma membrane and leakage of the cell content (16).

The pancreatic beta cell is thought to be particularly sensitive to apoptotic stimuli due to the inherent features of the specialized beta cell phenotype. It has been proposed that beta cell differentiation signals (such as Pdx-1), glucose metabolism, calcium handling as well as regulation of naturally occurring inhibitors of cytokine signaling contribute to sensitize the beta cell to apoptotic stimuli (17). Accumulating evidence suggests that apoptosis is the main form of beta cell death in both types of diabetes. It is widely accepted that apoptosis is likely to be the main form of beta cell death in type 1 diabetes (18; 19). It has been debated whether the reduced beta cell mass in type 2 diabetes is due to an increased loss of beta cells and/or to inadequate beta cell expansion to compensate for insulin resistance and/or beta cell dysfunction. Now, there is increasing evidence indicating that decreased beta cell mass in obesity-linked type 2 diabetes is above all caused by an increase in beta cell apoptosis, and that altered rates of beta cell replication or neogenesis play minor roles (9). It has been shown that elevated glucose concentrations directly induce beta cell apoptosis in human islets (20) and in cultured islets from diabetes-prone Psammomys obesus, a rodent with a natural tendency towards diet-induced type 2 diabetes (21). Cell apoptosis has also been shown to be the mechanism leading to beta cell loss in the Zucker diabetic fatty (ZDF) rat, another model of type 2 diabetes. In ZDF rats, hyperglycemia is detected when the expansion of the beta cells no longer compensates the degree of insulin resistance and beta cell apoptosis (22; 23). The involvement of apoptosis in type 2 diabetes has been reinforced by two recent studies performed with human autopsy specimens (24; 25). In particular, Butler and colleagues (25) have reported a deficit in relative beta cell volume in type 2 diabetic patients compared to non-diabetic weight-matched controls and an increased frequency of beta cell apoptosis. It was observed that the frequency of beta cell regeneration is very low, and that it may not contribute significantly to beta cell mass in humans (25). However, these data are difficult to interpret since they are “snapshots” taken at the time of autopsy. Furthermore, apoptotic cells are rapidly cleared from tissues in vivo, rendering an accurate quantification of apoptosis difficult to perform *in vivo* (26). In addition, as these studies were performed on autopsy specimens, and prospective studies are not possible to perform in humans (because of lack of non-invasive means to measure the beta cell mass), it is still unknown what the beta cell mass was *before*

the onset of diabetes. Consequently, it remains to be established whether apoptosis is one of the triggers leading to the onset of diabetes, or whether it occurs at the late stages of the disease in humans.

Which factors could contribute to the increased rate of apoptosis observed in type 2 diabetes? It has been proposed that cytokines, lipids (lipotoxicity), glucose (glucotoxicity), as well as a synergic combination of glucose and lipids (glucolipotoxicity) are the main stimuli leading to beta cell apoptosis in the context of type 2 diabetes (12; 27-30). In addition, islet amyloid polypeptide (IAPP) and subsequent amyloid fibril formation (28), ER-stress (31), inadequate levels of circulating growth factors (8; 31) and signaling factors from the adipocytes are other possible causes for the decreased beta cell mass in type 2 diabetes (12).

Obesity is a typical condition that leads to an insulin-resistant condition. This increased insulin demand is often compensated by an increase in beta cell mass. However, in some cases (about one-third of obese subjects), the compensatory beta cell mass and/or function is inadequate (9). It has been proposed that although there may be an initial compensatory increase in beta cell mass, the onset of type 2 diabetes in both human and rodent models is accompanied by a progressive decrease in beta cell mass, presumably due to increased circulating glucose and/or free fatty acid concentrations (8; 32). This beta cell loss arises from a marked increase in beta cell apoptosis, which far outweighs modest increases in beta cell replication and neogenesis (8; 25). This loss of beta cell mass may subsequently induce further dysfunction of the residual beta cells, possibly due to overstimulation of these beta cells or chronic hyperglycemia and/or hyperlipidemia (12), thus further worsening the condition of the patient. Eventually, in the most severe cases, a “point of no return” due to the loss of beta cell mass can be reached and a permanent type 2 diabetic state arises that must ultimately be treated with insulin replacement therapy. Therefore, maintaining beta cell survival and adequate beta cell mass appears to be one potential strategy to prevent and/or treat type 2 diabetes (9).

2. The Extracellular Matrix (ECM)

2.1. General introduction on extracellular matrix (ECM)

Until two decades ago, the ECM was viewed as a passive and inert scaffold stabilizing the physical structure of tissues (33). Indeed, attachment of cells to ECM is crucial for maintenance of tissue integrity. However, it has recently become clear that ECM is an extremely dynamic structure and that it is an essential regulator of primordial biological functions. Engagement of cells with ECM proteins is crucial for various biological processes, including cell adhesion, spreading, proliferation, differentiation, apoptosis, and gene induction, contributing to maintenance of tissue integrity, embryogenesis, wound healing, and the metastasis of tumour cells (34-37). The importance of the ECM is underscored by the existence of many genetic and autoimmune diseases that perturb ECM structure or the adhesion of cells to the ECM in humans (38). ECM remodeling is involved in many diseases including the development and progression of malignant tumours (39) and diabetic nephropathy and microangiopathy (40).

There are two main types of ECM in the tissues: the basal lamina contained in the basement membrane, a condensed layer that is formed adjacent to epithelial cells, and the interstitial matrix that is present in connective tissues (Fig. 2). Basement membranes cover the basal surfaces of virtually all epithelia, surround the surfaces of muscle fibers, and ensheath nerves. Basement membranes are comprised of two, sometimes three distinct layers. Two layers are comprised in the basal lamina, which is produced by epithelial cells: lamina lucida adjacent to the plasma membrane of cells and lamina densa just below. In some cases there is a third layer (reticular lamina) that connects the basal lamina to the connective tissue. The basal lamina contains a variety of adhesive glycoproteins, including collagen IV, laminin, fibronectin and proteoglycans (33; 41; 42). Interaction of these ECM proteins with the cells is mediated by transmembrane receptors, of which the integrins constitute the most important class.

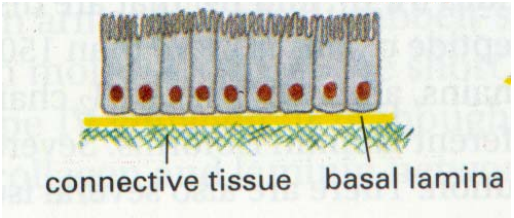

Fig. 2: The basal lamina and the connective tissue underlying an epithelial cell sheet. Reproduced and modified from reference (42).

2.2. ECM receptors: Integrins

Cell adhesion to ECM is mainly mediated by heterodimeric transmembrane receptors belonging to the integrin family (34). Integrins are a part of a bridge between ECM and cytoskeletal proteins at sites of tight juxtaposition between the cell surface and ECM (43). These sites of adhesion include focal adhesions (also called focal contacts), fibrillar adhesions and focal complexes (44). Integrins are heterodimers that include α and β subunits with each subunit having an extracellular domain, a single transmembrane region, and (other than β4) a rather short cytoplasmic domain associating with the actin cytoskeleton and affiliated proteins (34), (Fig. 3).

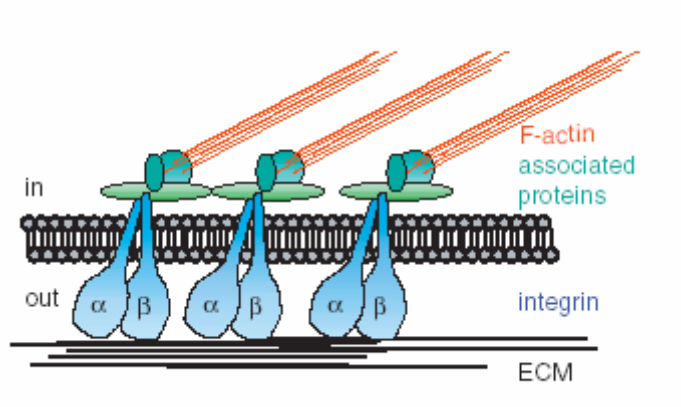

Fig. 3: Schematic representation of a cell-ECM adhesion site. Integrins connect ECM with the cell interior, where they are in contact with adaptor/signaling proteins and the actin cytoskeleton (reproduced from reference (45)).

The vertebrate integrin family includes at least 18 distinct α subunits and 8 or more β subunits; these can associate to form over 20 distinct integrin heterodimers (34). The ligand binding abilities of the integrin heterodimers are determined by the α/β pairings (Fig. 4). The usual ligands for integrins are the ECM proteins collagen, laminin, vitronectin, and fibronectin (33). Some integrins, such as α5β1, the “classic” fibronectin receptor, interact only with a single ECM protein; however, more commonly, a given integrin will recognize several distinct ECM proteins (34). Genes for the β subunits and for all but four of the α subunits have been knocked out and each phenotype is distinct, reflecting the different roles of the various integrins (46). Numerous and various biological defects have been observed in these knock-out mice, including peri-implantation, embryonic and perinatal lethality, major developmental defects and abnormal basement membrane assembly (46). However, tissue-specific and/or conditional integrin knock-out models have been under-exploited as tools to evaluate further the role of specific integrins in a given cell-type or tissue function in the adult.

Integrin occupancy and clustering initiate not only adhesion and cytoskeletal organization via direct physical interactions of integrins with other proteins, but also activate many intracellular pathways that regulate cell growth, migration, polarity, survival, differentiation and gene expression (36). This signaling from engaged integrins to the cell interior is an

important aspect of outside-in signaling. Conversely, the capacity of integrins to bind their ligands is regulated by cellular signaling mechanisms through a process often called inside-out signaling (44). Integrins are therefore signaling receptors that transmit information in both directions across the plasma membrane. In addition, integrins can act as essential cofactors for growth factor signaling. Indeed, in many cases growth factor signaling does not occur unless cells are adherent to ECM (47; 48).

The β1 integrins are expressed on most epithelial cells, where they bind collagen, fibronectin, laminin-1 and laminin-5. They have been suggested to play important roles in morphogenesis (49; 50), regulation of proliferation (51) and cell survival (52; 53). Within islets, it has been shown that the laminin-5 receptors α3β1 and α6β1 integrins are expressed on beta cells (5; 54).

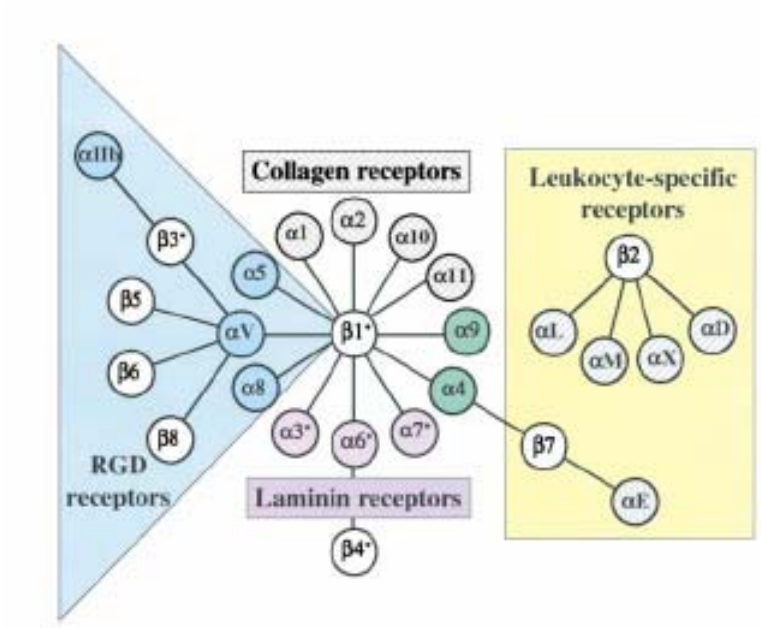

Fig. 4: The integrin receptor family. This figure shows the mammalian integrin subunits and the binding specificity of the given α/β heterodimer. Reproduced from reference (34).

2.3. ECM and cell survival

2.3.1. Anoikis

Apoptosis can be induced by numerous triggers, one of them being the loss of cell anchorage. Anoikis is a Greek word meaning “homelessness” (55) and is defined as apoptosis that is induced by inadequate or absent cell-ECM interactions (56). The role of cell anchorage for cell survival has been demonstrated and studied in several cell types, including the pancreatic islet cell (57; 58). It has been reported that basement membrane ECM, but not fibronectin or collagen suppresses apoptosis of mammary epithelial cells (59), indicating that for a given cell type only specific ECM components can promote cell survival. This specificity for a given ECM component has since been confirmed for different cell types, including for the pancreatic beta cell (60).

Signaling pathways involved in apoptosis usually converge on a common machinery of cell destruction that is activated by a family of cysteine proteases: caspases (15; 61). Caspases are activated during most forms of apoptosis. They are divided into two major subgroups, the initiator caspases and the executioner (or effector) caspases. The initiator caspases (including caspases -2, -8, -9 and -10) contain long prodomains and interact with death domain (DD)-containing adaptor proteins, bringing them into close proximity with death receptors. The executioner caspases (which include caspases -3, -6 and -7) mainly function by proteolytic fragmentation of structural and functional protein substrates, leading to dismantling of the cell architecture as well as the initiation of DNA-fragmentation, the end-point of the apoptotic process.

As in most apoptotic pathways, anoikis is thought to be mediated by the activation of caspases. Caspase-8 activity has been shown to be induced by cell detachment (62-64). One of the major questions in anoikis research is how the caspase cascade is initially activated by simple detachment of cells from the ECM (56). Recently, it has been proposed that activation of Fas signaling complex leading to activation of caspase-8 plays an important role during anoikis (62-64). Another possible mechanism leading to activation of caspase-8 during anoikis is integrin-mediated death (IMD) (65). This is a mechanism whereby unligated integrins in cells adhering to substrates devoid of appropriate ECM ligands are proposed to directly recruit caspase-8 to the plasma membrane independent of death receptors, leading to the activation of the cascade caspase and ultimately to anoikis (65; 66). In summary, it appears that caspase-8 activation can be induced by cell detachment from ECM as well as by interaction of the cell with inappropriate ECM.

2.3.2. Pro-survival signaling and integrins

It was first proposed in 1992 that cells require survival signals to avoid their engagement into apoptosis (67). This concept has been reinforced since by numerous observations assessing pro-survival effects of different proteins such as growth factors and hormones. How do adequate ECM-cell interactions promote cell survival? The involvement of integrins in the pro-survival signaling emanating from the ECM is now well established (66). Meredith et al. were the first to demonstrate that contact of cells with anti-β1 integrin antibodies inhibits apoptosis induced by the absence of ECM (53). Many groups have since reported the importance of β1 integrin subunits in mediating survival signals emanating from the basement membrane (which is a complex ECM composed of laminin, collagen IV, nidogen and perlecan) in mammary epithelial cells (52; 59; 68; 69). Interestingly, it was observed that only normal but not tumor mammary epithelial cells are dependent on β1 integrin signaling for survival (52). Different α integrin subunits have also been shown to play a role in mediating ECM-dependent survival (70-72), including the α6 integrin subunit (69). It appears that different cell types have their own favored ECM for protection from apoptosis, depending on the repertoire of integrins expressed on their cell surface (73). Transgenic mice carrying a targeted deletion of the C-terminal segment of the β1 integrin subunit cytoplasmic domain have been generated (74). Embryonic fibroblasts derived from the homozygotes carrying this deletion display disrupted fibronectin-induced survival, thus supporting the concept that integrins play an essential role in pro-survival signaling emanating from ECM (74; 75).

In summary, the concept that ligation of integrins to cognate ECM ligand can induce pro-survival signaling in normal cells is now generally accepted. In the next section, the current state of knowledge on the role of ECM and integrins in the context of beta cell survival and function is discussed.

2.4. ECM and the pancreatic beta cell

Studies assessing the impact of ECM on beta cell development and function are limited in number but provide important evidence that it can influence many aspects of beta cell biology including survival, proliferation, differentiation and insulin secretion. ECM is thus considered as one important component of the islet microenvironment. Islet basement membrane has been reported to be composed of collagen IV and laminin (2). Within islets, the laminin-5 receptors $\alpha3\beta1$ and $\alpha6\beta1$ integrins (76; 77) are expressed in pancreatic adult rat beta cells (5; 54). As described above, both type 1 and type 2 diabetes are characterized by reduced islet beta cell mass and impaired beta cell function. We will therefore go deeper into what is known about the effects of the ECM on the survival and the function of the pancreatic beta cell.

2.4.1. ECM and pancreatic beta cell survival

Transplantation of islets of Langerhans represents a viable therapeutic approach for the treatment of type 1 diabetes. Unfortunately, several problems can occur after allogeneic islet transplantation: primary non-function, rejection, the recurrence of autoimmune disease, as well as poor rate of survival of insulin-secreting cells before and after transplantation (78). In addition, the immunosuppression treatment following the transplantation process may itself impact negatively on the function and survival of transplanted islets.

The causes of this poor rate of survival are not well understood. The procedure leading to pancreatic beta cell isolation involves disruption of cell-cell and cell-ECM contacts (1; 58; 79). It is suspected that the loss of ECM during the isolation process leading to cell death by anoikis might be one of the causes for the poor survival rate of the transplanted islets (1; 57; 58; 79). The beneficial effect of ECM on the survival of islet cells has been suggested by several groups (57; 58; 60). There is evidence indicating that ligation of integrins to proper ECM proteins is necessary for survival of pancreatic beta cells in culture (60). However, signaling pathways involved in this improved survival induced by the contact of islet cells with the ECM have not yet been explored.

2.4.2. ECM and pancreatic beta cell function

Beta cells isolated from islets of Langerhans display altered glucose-induced insulin secretion when maintained for several days in culture. Several groups have shown that long-term culture of islet cells on different ECM (fibronectin, collagen, or bovine corneal endothelial cell matrix (BCEM)) improves their secretory capabilities compared to culture in suspension or on control substrates (non-coated plastic dishes or poly-L-lysine (pLL)-coated dishes) (58; 80-83). Furthermore, it has been observed that incompletely digested, ECM-embedded islets display improved insulin secretion in response to glucose compared to well digested islets

(without ECM), suggesting that appropriate ECM-islets interactions are required for optimal islet function (57).

The 804G extracellular matrix (804G-ECM, an ECM produced by the rat bladder carcinoma cell line 804G) is rich in laminin-5 (epiligrin) and also contains fibronectin (84-86). It has been reported that the 804G-ECM is able to induce attachment and spreading of many epithelial cell types, including pancreatic beta cells (85; 87). Short-term culture (a few hours) on 804G-ECM improves glucose-stimulated insulin secretion of isolated beta cells compared to control condition (beta cells grown on plastic dishes or glass) (5). It has been suggested that the effects of 804G-ECM on beta cell spreading is mediated, at least in part, by engagement of laminin-5 to $\alpha 6\beta 1$ integrins (5). However, the downstream signaling pathways involved in the effects of the 804G-ECM on the beta cell function have not yet been identified.

3. Summary and general aims of the thesis project

It has recently emerged that cell death by apoptosis is involved in the development of not only type 1 diabetes, but also of type 2 diabetes, although to different extents. Therefore, studies aimed at understanding the mechanisms controlling pancreatic beta cell survival are an important area in diabetes research. It has been shown in numerous cell types that ligation of integrins to cognate ECM ligand is important for cell survival. Furthermore, several studies have suggested that ECM is an important factor in maintaining survival of pancreatic beta cells as well. However, the mechanisms involved in the pro-survival effects of the ECM on the pancreatic beta cell were completely unexplored at the beginning of this thesis project.

As introduced in the next part, a number of reports have shown that engaged integrins can activate several signaling pathways that are able to mediate its pro-survival effects. These signaling pathways involve the kinases Focal Adhesion kinase (FAK), phosphatidylinositol 3-kinase (PI3K), protein kinase B (PKB/Akt, hereafter called Akt), and the Mitogen-activated protein (MAP) kinase ERK. However, the relevance of a given signaling molecule in mediating the pro-survival signaling induced by ECM appears to be cell – type specific. It was therefore asked which of these intracellular signaling pathways might be involved in the effects of ECM on pancreatic beta cell survival.

In our laboratory, it has been shown that primary rat pancreatic beta cells express the laminin-5 receptors $\alpha 3\beta 1$ and $\alpha 6\beta 1$ integrins (5; 54). Furthermore, it has been found that the 804G-ECM – a laminin 5-rich ECM - induces spreading and improves glucose-stimulated insulin secretion of primary rat pancreatic beta cells (5), indicating that this ECM most probably interacts with integrins expressed on the rat beta cells. Therefore, it appeared that the 804G-ECM should be an appropriate model to study the effect of the ECM on primary pancreatic beta cell survival and the mechanisms involved. In addition, we chose to study primary cells and not cell lines, as the pattern of expression of integrins as well as the dependence of the cell on the presence of a cognate ECM for survival can differ considerably between primary cells and cell lines (transformed or not). Therefore, the general aims of this thesis work were to answer the following questions:

1) Does the 804G-ECM improve primary pancreatic beta cell survival?

2) Is the 804G-ECM able to activate signaling pathways and if so, are they involved in the pro-survival effect of the 804G-ECM?

3) Is the 804G-ECM able to induce a gene expression program leading to altered function and/or survival of the pancreatic beta cell?

This thesis is composed by two reports (“ Paper 1” and “Paper 2”) accepted for publication in peer-reviewed journals. The next part of this thesis (Part II, Paper 1) focuses on the two first points 1) and 2), while Part III (Paper 2) is mainly devoted to the point 3).

PART II (PAPER 1):

ACTIVATION OF KINASES BY ECM AND PANCREATIC BETA CELL SURVIVAL

1. Introduction to Part II (Paper 1)

1.1. Pro-survival signaling from ECM : an overview

Engagement of integrins by cognate extracellular ligands leads to activation of several pathways involving an astonishing number of signaling and/or adaptor proteins ((36; 88), Fig. 5 and Fig. 7), that control many aspects of cellular responses to growth and survival factors. An early insight into a possible signaling via integrins was provided by the observation that integrin-mediated adhesion and/or clustering of integrins lead to enhanced tyrosine phosphorylation (89). It soon became clear that adhesion was activating a cytoplasmic non-receptor tyrosine kinase now known as FAK (90; 91). In addition to FAK, integrin binding is thought to be involved in the regulation of activity of a multitude of signaling molecules, including PI3K, protein kinase B (PKB/Akt) and the mitogen activated protein (MAP) kinase ERK (92-94). These proteins have all been implicated in integrin-mediated protection of cells against various apoptotic stimuli and in control of gene expression. In the next sections, we will go deeper into what is known about these proteins in the context of ECM-induced cell survival.

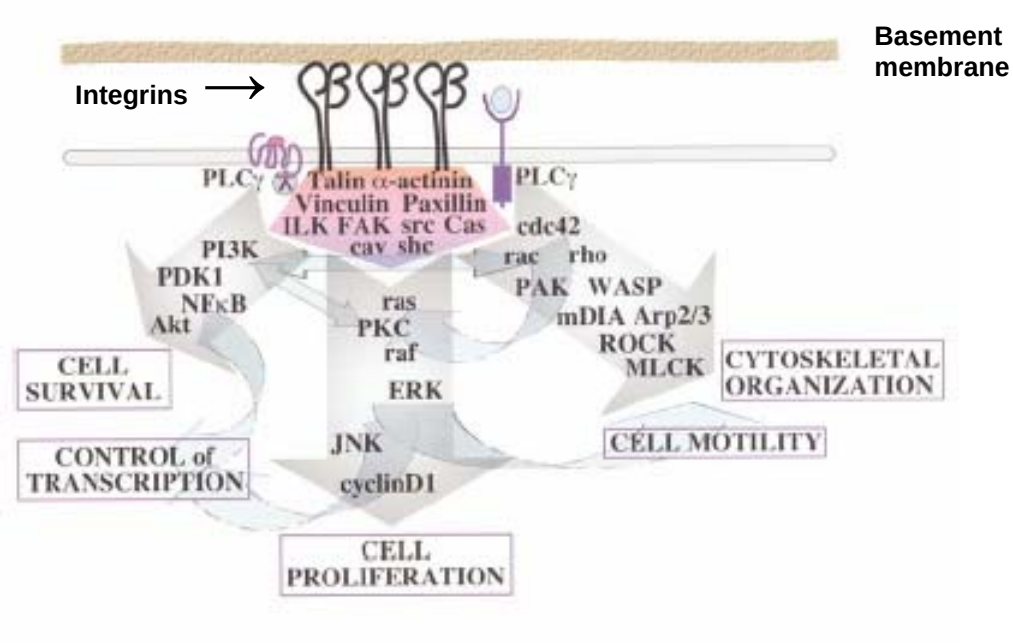

Fig. 5: General outline of integrin signaling and its biological outcomes. This figure shows some major signal transduction pathways and their key players that lead to some major effects of ECM-integrin interactions on cell survival, proliferation, motility and cytoskeletal organization (reproduced and modified from reference (34)).

1.2. Focal Adhesion kinase (FAK)

1.2.1. Activation of FAK by integrins

The cloning and characterization of FAK were reported in 1992 (90; 91). FAK is a non-receptor tyrosine kinase, which consists of a central tyrosine kinase (catalytic) domain flanked by large N- and C-terminal non-catalytic domains (91; 95). The N-terminal region contains a FERM domain that is thought to mediate interactions of FAK with proteins associated with the plasma membrane. The C-terminal domain of FAK, designated "FAT", for Focal Adhesion Targeting, is essential for localizing FAK to integrin-adhesion sites (92; 95). Finally, two proline-rich motifs (PR1 and PR2) are located between the kinase and the FAT domains (Fig. 6). These motifs mediate interactions with Src-homology 3 (SH3)-domain-containing proteins. These functional and structural characteristics of FAK have led to its definition as a "scaffolding protein with kinase activity".

FAK is localized in focal adhesions (90; 91), and is widely expressed in different tissues (90). The first indication that FAK is a signaling protein was provided by the demonstration that it is phosphorylated on tyrosine residues when cells are plated on the ECM molecule fibronectin (90; 96; 97). It was subsequently reported that other ECM proteins (laminin, collagen IV and vitronectin) also induce tyrosine phosphorylation of FAK (89; 98). In addition, the overall phenotype of the FAK knock-out mouse is similar to the fibronectin knock-out mouse (99; 100), reinforcing the concept that FAK is implicated in signaling induced by engagement of cells to ECM.

Upon integrin ligation and clustering, FAK is phosphorylated at Tyr-397 as well as at several additional sites within the kinase and the C-terminal domains (101-103) (Fig. 6). Tyr-397 is a major site of FAK auto-phosphorylation both *in vivo* and *in vitro* (104), and phosphorylation at Tyr-397 correlates with increased catalytic activity of FAK (101; 105). Therefore, phosphorylation of FAK at Tyr-397 is considered as a hallmark of integrin signaling.

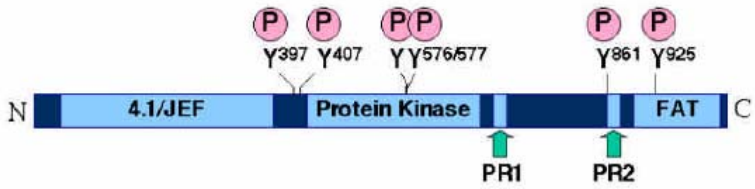

Fig. 6: Linear structure of FAK showing its major domains, motifs and sites of tyrosine phosphorylation. The 4.1/JEF domain is also called FERM domain, and is thought to mediate interactions with proteins associated with the plasma membrane. The two proline-rich motifs mediate interactions with SH3-domain-containing proteins. Reproduced and adapted from reference (95).

1.2.2. *FAK and downstream signaling*

FAK interacts with a number of cytoskeletal and signaling proteins, including Src, PI3K, Grb2, $p130^{Cas}$ and paxillin (95; 106), (Fig. 7). The finding that FAK is phosphorylated when cells are plated on fibronectin led to the hypothesis that FAK might transmit a signal within the cell through association with, and phosphorylation of, proteins that contain Src homology 2 (SH2) domains (90). The SH2 domain is a conserved region of about 100 amino acids, which specifically and selectively binds phosphotyrosyl proteins and promotes intra- and intermolecular protein-protein interactions (107). Indeed, it was subsequently demonstrated that phosphorylation of residue Tyr-397 enables the recruitment of several signaling proteins harboring SH2 domains. One such protein is the p85 kDa subunit of PI3K (108-110), which recruitment and activation leads to the activation of the kinase Akt. Another example is Src kinase (104). Src kinase then mediates phosphorylation on other sites on FAK, creating additional SH2-binding sites (101; 102). One such site is the phosphorylated residue Tyr-925 that can bind the SH2-domain of Grb2, leading to the activation of the MAP kinase ERK cascade (102; 111). Activation of these pathways involving the signaling proteins PI3K-Akt and ERK by engaged integrins is one of the main themas of this thesis project, and this topic is described more in detail below.

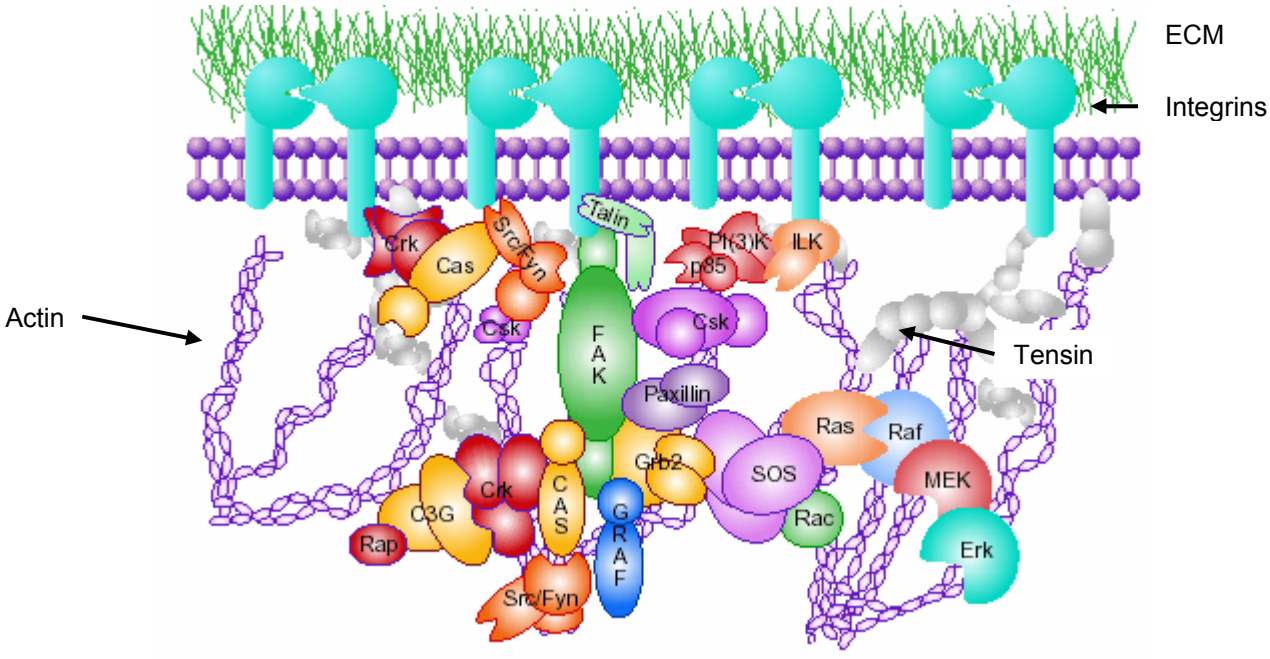

Fig. 7: Signaling complexes associated with, or activated by, integrins. FAK binds to and recruits additional signaling molecules, creating a complex signaling network that is intimately connected with the actin cytoskeleton network. Reproduced and adapted from reference (88).

1.2.3. Functions of FAK in the control of spreading and survival of cells

It is now acknowledged that integrin-induced FAK activation is involved in the regulation of cell proliferation (112-114), cell spreading/motility and cell survival (92; 93; 95).

Fibroblasts derived from FAK knock-out mice display deficient integrin-triggered spreading and migration (100). By contrast, overexpression of FAK either in CHO cells (115) or in FAK-deficient fibroblasts (116; 117) leads to increased spreading and/or migration of cells on fibronectin (115-117). Interestingly, overexpression of a FAK mutant lacking the Tyr-397 autophosphorylation site fails to rescue the effects of the inhibition or absence of FAK on cell spreading and/or migration (115-118). This suggests that auto-phosphorylation of FAK on residue Tyr-397 plays a crucial role in ECM-induced cell migration. Since, numerous studies have implicated FAK as a positive regulator of cell spreading and migration.

One of the main functions of FAK is the protection of cells against apoptosis. It was first reported that tyrosine phosphatase inhibitors block apoptosis subsequent to detachment from ECM (53). Subsequently, Frisch et al. (119) showed that constitutively active FAK mutants rescue MDCK cells from anoikis. Conversely, attenuation of FAK expression by treatment with antisense oligonucleotides induces apoptosis of tumour cell lines (120) and microinjection of peptides blocking binding of FAK to endogenous integrins or of anti-FAK antibody induces apoptosis of fibroblasts (121). It has also been reported that FAK-deficient cells are more susceptible towards serum withdrawal-induced apoptosis (122), and different dominant-negative FAK mutants have been shown to induce apoptosis in absence (122) or in presence of serum (123; 124). Now, it is generally accepted that FAK plays a critical role in suppressing anoikis and apoptosis caused by diverse inducers. Interestingly, it has been shown by several groups that FAK mutated on residue Tyr-397 is unable to promote survival of cells (119; 125; 126), suggesting that phosphorylation of Tyr-397 plays a critical role also in the pro-survival function of FAK. However, it has also been reported that the survival signal from the $\alpha 5\beta 1$ integrin (in CHO cells overexpressing integrin $\alpha 5$ subunit) does not correlate with increased FAK phosphorylation (71), suggesting that pro-survival signaling from integrins may in some contexts occur independently of FAK.

In summary, FAK and phosphorylation of its tyrosine residue Tyr-397 have been shown to play central roles in integrin signaling leading to regulation of cell migration and survival. Several proteins have been involved in this signaling, including PI3K, Akt and ERK.

1.3. The PI3K-Akt signaling module

1.3.1. Phosphatidylinositol 3-kinase (PI3K)

There are multiple isoforms of PI3Ks in mammalian cells, and the best characterized is class I. Class I PI3Ks comprise a p110 catalytic subunit and a regulatory adapter subunit: p85. The p85 subunit contains an SH3 domain and two SH2 domains (127; 128). These SH2 domains allow the interaction between p85 and proteins containing specific tyrosine-phosphorylated sequences, such as the ones present on phosphorylated FAK proteins. This process recruits the p110 catalytic subunit to the plasma membrane, where it can phosphorylate phosphatidyl inositol-4,5-biphosphate [$PI(4,5)P_2$] and thereby generate phosphatidyl inositol-3,4,5-triphosphate [$PI(3,4,5)P_3$] (129). This phosphorylated lipid is implicated in signaling pathways involved in the regulation of cell survival, gene expression and cell metabolism, as well as cytoskeletal rearrangements (130; 131). It has been shown that the PI3K-generated [$PI(3,4,5)P_3$] lipids recruit signaling proteins by direct binding to their PH domains, and either allosterically modify their activity or induce relocalization of the protein to defined areas of the plasma membrane, where activation can occur (132). Such signaling proteins include the serine-threonine kinase Akt (133; 134). Not all but a major part of PI3K signaling is mediated via Akt. The alternative signaling pathways mediated by PI3K are out of the scope of this introduction and are reviewed by Downward (135).

1.3.2. Protein kinase B (PKB/Akt)

In 1991, several groups independently identified the serine/threonine kinase Akt (also called PKB) (136-138). One of these groups identified this kinase as being the product of the oncogene v-*akt* of the acutely transforming retrovirus AKT8 found in a rodent T-cell lymphoma (136). Three isoforms of Akt exist in mammalian cells: Akt1, Akt2 and Akt3. These three Akt proteins, although encoded by distinct genes localized on different chromosomes, have approximately 80% amino acid identity and similar domain structures (139). Akt1 and Akt2 both show a wide distribution of expression, however their levels of expression differ (140; 141). Akt family proteins contain a central kinase domain with specificity for serine or threonine residues in substrate proteins (136; 137; 141; 142). In addition, the amino terminus of Akt includes a pleckstrin-homology (PH) domain which mediates lipid-protein and/or protein-protein interactions (141; 142). Akt has multiple effects on cell survival, metabolism and proliferation (134; 135).

Activation of Akt requires its translocation to the plasma membrane and phosphorylation of Akt is also a critical step leading to its activation (143; 144). Mutation of the residues Thr-308 and/or Ser-473 alters activation of Akt, suggesting that phosphorylation of these two residues is required for maximal activity (145). Akt binds specifically to PI3K-generated [$PI(3,4,5)P_3$] lipids, but these do not directly affect its activity (146). A model where

PI3K-generated [PI(3,4,5)P_3] lipids may recruit Akt to the plasma membrane, where it is phosphorylated and activated by a membrane-associated protein has been proposed by Alessi and colleagues (145; 146). This model has been reinforced by the isolation of this putative [PI(3,4,5)P_3]-dependent protein kinase (PDK-1) that phosphorylates and activates Akt on residue Thr-308 (147). The putative kinase(s) or mechanism(s) responsible for Ser-473 phosphorylation have not yet been clearly identified (148). Nevertheless, several candidates have been proposed to be Ser-473 kinases, including integrin-linked kinase (ILK) (149).

In summary, Akt is activated (indirectly) by the PI3K-generated [PI(3,4,5)P_3] lipids, and its activity is thus sensitive to the specific PI3K inhibitors Wortmannin and LY294002 (150). Full activation of Akt requires phosphorylation at the residues Thr-308 and Ser-473.

1.3.3. Activation of the PI3K-Akt pathway by ECM

It is now known that a diverse array of physiological stimuli, including ECM, are capable of inducing Akt kinase activity, primarily in a PI3K-dependent manner (139). Diverse ECM molecules (including fibronectin, laminin and collagen) and integrin subunits have been shown to induce PI3K and/or Akt activities. As mentioned above, the p85 subunit of PI3K can associate directly with FAK in response to cell adhesion to the ECM protein fibronectin (151). Furthermore, it was shown in the same report that PI3K activity associated with FAK increases upon attachment to fibronectin and that this is concomitant with increased FAK tyrosine phosphorylation (151), providing a first indication that PI3K might be involved in the signaling downstream of cell adhesion-induced FAK activation. The same group subsequently reported that phosphorylation of FAK on residue Tyr-397 is required for its binding to PI3K and for the activation of PI3K (108). Furthermore, adhesion of cells to fibronectin leads to accumulation of PI3K-generated phosphorylated lipids, resulting in activation of Akt kinase activity (152). This fibronectin-induced activation of Akt is dependent on PI3K activity (152). Generation of primary fibroblasts carrying a targeted deletion of the segment of β1 integrin cytoplasmic domain required for activation of FAK has been reported (74). These fibroblasts have impaired FAK-PI3K-Akt signaling in response to adhesion to fibronectin (75). Therefore, it can be concluded that engagement of integrins to cognate ECM can activate the PI3K-Akt signaling pathway.

1.3.4. Role of the PI3K-Akt pathway in cell survival

Yao and Cooper provided the first evidence indicating that PI3K is implicated in the suppression of apoptosis (153). This report demonstrated that inhibition of PI3K activity using two pharmacological inhibitors of PI3K (LY294002 and Wortmannin) abrogates the ability of NGF to promote cell survival. Since, PI3K activity has been found to be required for growth factor-dependent survival of a wide variety of cultured cell types ranging from fibroblasts to neurons (139). The observation that Akt is a target of PI3K, together with the finding that PI3K

mediates growth factor-dependent survival, suggested that Akt might be a critical regulator of cell survival. This hypothesis has been confirmed by many groups (as reviewed by (139)), leading to the conclusion that the PI3K-Akt pathway is one of the major anti-apoptotic pathways operating in cells (139). In addition, it has been shown that specific deletion of Akt1 leads to partial neonatal mortality and the surviving Akt1 knock-out animals are smaller in size than control mice, suggesting that Akt1 may be involved in regulation of survival and/or proliferation of cells (154; 155). Several effectors downstream of activated Akt have been reported to mediate its survival signaling. For example, Akt has been shown to directly phosphorylate the pro-apoptotic Bcl2-family member Bad, leading to its binding to the 14-3-3 protein and thus preventing it from exerting its pro-apoptotic effects (156-158). Furthermore, Akt has been reported to regulate the activity of transcription factors such as members of the Forkhead family of transcription factors and NF-κB (159; 160), leading to altered gene expression. However, it seems that these effects of Akt on the activity and/or expression of proteins regulating apoptosis are cell-type and/or context-dependent.

The PI3K-Akt pathway was first reported to mediate ECM-induced survival of normal epithelial cells in a study showing that overexpression of constitutively active PI3K and Akt mutants are able to protect MDCK cells against anoikis (161). Since, several groups have confirmed that the PI3K-Akt pathway can mediate the pro-survival signaling from ECM and/or integrins (69; 72; 162). The induction of the PI3K-Akt pro-survival signaling by ECM and/or integrins has been reported to occur specifically on a given ECM substrate or through a specific integrin subunit, and this specificity is cell type-dependent (72; 73). For example, it has been shown that overexpression of the α5 integrin subunit protects epithelial cells against serum deprivation-induced apoptosis through the PI3K-Akt pathway, whereas overexpression of α2 subunit does not (72). The pro-survival signaling emanating from ECM-integrin interaction or FAK activation does not always involve PI3K-Akt pathway (122; 123). Indeed, other pathways such as JNK (123) and MAPK-ERK (73) have also been reported to mediate the pro-survival signaling from ECM.

In summary, it has been shown that ECM and/or engagement of integrins to cognate ECM proteins can activate the PI3K-Akt survival signaling pathway. However, as discussed below, other pathways have also been shown to mediate the pro-survival signaling from ECM, such as the MAP kinase ERK pathway.

1.4. The MAP kinase ERK pathway

1.4.1. General introduction

The MAP kinases comprise a large family of signal-regulated serine/threonine kinases. Three main MAP kinases cascades have been characterized: the extracellular-regulated kinases 1 and 2 (ERK1/2) pathway, the-Jun N-terminal kinases/stress-activated protein kinases (JNK/SAPK) cascade and the p38 pathway (reviewed by Pearson, 2001 #453}). The MAP kinase ERK pathway can be activated by many different stimuli, including growth factors, cytokines, and ECM. This pathway has been implicated in a wide range of cellular functions, including actin cytoskeleton reorganization, migration, cell proliferation, and survival (163), (reviewed by (164-166)).

The “classic” ERK cascade begins with the activation and autophosphorylation of RTK (receptor tyrosine kinase) on the cell surface (167). This creates phosphotyrosyl binding sites for SH2-domain adaptor proteins such as Grb2 and Shc, which in turn can recruit other proteins such as Sos, a partner of Grb2 that is an exchange factor for Ras GTPase. Activation of Ras at the membrane by Sos leads to recruitment of Raf-1 (also called MAPKKK) into the membrane and its subsequent activation. Raf-1 subsequently phosphorylates and activates MEK1 and MEK2 (also called ERK kinases, or MAP kinases kinases, MAPKK), which then can phosphorylate ERK1 and ERK2 (also called p44 and p42, respectively) on two residues (threonine and tyrosine) leading to their activation (reviewed by (164; 168; 169)). Once activated, ERK1/2 can target a multitude of proteins, including membrane proteins (such as phospholipase A2) and cytoplasmic proteins (including downstream kinases, such as ribosomal S6 kinases). Activated ERKs can also can translocate into the nucleus where they phosphorylate transcription factors, thereby regulating their activity (reviewed by (164; 169)).

1.4.2. Activation of the MAP kinase ERK pathway by ECM

ECM can activate the MAPkinase ERK pathway by two main mechanisms. First, it has been reported that activation of ERK by serum or by growth factors is strongly dependent on adhesion to ECM proteins (reviewed by (94; 168). The ability of integrin-mediated adhesion to regulate the efficiency of the growth factor activation of ERK has been observed in countless cell and tissue types (94). Second, ECM has been shown to directly activate the ERK pathway. Indeed, it has been shown that adhesion of fibroblasts onto different ECM (including fibronectin, laminin, vitronectin and collagen type IV) can lead to activation of the ERK pathway (109; 170; 171). Mainiero and colleagues have shown that ligation of $\alpha 6\beta 4$ to laminin-5 leads to tyrosine phosphorylation of Shc, recruitment of Grb2, activation of Ras leading to stimulation of the ERK pathway (172). This was specific for $\alpha 6\beta 4$, as ligation of $\alpha 3\beta 1$ and $\alpha 2\beta 1$ or collagen did not cause these events (172), indicating that only ligation of specific integrins can activate the ERK pathway in a given cell type.

How does ligation of integrins to cognate ECM ligand lead to activation of ERK? Different mechanisms have been proposed, highlighting the complexity of integrin signaling (reviewed by (88)). For example, the role of FAK in ECM-induced ERK activation is controversial: FAK is capable of activating MAPK through recruitment of Grb2 (102), Shc or Src (173; 174), but other mechanisms independent of FAK that result in ERK activation have also been described (reviewed by (88; 94)). In addition, paradoxical effects of the PI3K-Akt pathway on ECM-induced activation of the ERK pathway have also been reported. For example, it has been shown that β1 integrin-induced ERK activation in monocytes is dependent on PI3K activity (175), while others have reported that PI3K-Akt and ERK pathways act independently from each other (176). It has been proposed that the role of FAK and/or of PI3K in integrin-induced ERK activation may be cell-type and/or context-dependent.

What are the consequences of adhesion-dependent ERK activation? It has been reported to affect numerous cellular functions, including regulation of gene expression, cell proliferation, survival and migration (as reviewed by (94)). The effect of ECM-induced ERK signaling on the regulation of cell survival is discussed below.

1.4.3. Role of the MAP kinase ERK pathway in cell survival

In general, ERK is considered to be a pathway leading to increased cell survival and protection against several different pro-apoptotic stimuli, including death receptor-mediated activation and survival factor removal (reviewed by (166)). Disruption of the ERK kinase MEK1 gene causes embryonic lethality (177). In addition, MEK1-deficient fibroblasts migrate more slowly than wild-type cells on a fibronectin substrate, but behave normally on a collagen substrate. These findings are consistent with a requirement for the ERK pathway for stimulation of migration by extracellular matrix and cell survival (177). However, ERK1 disruption causes minimal phenotypic changes, and has no effect on cell survival (178). Nevertheless, it is possible that ERK2 can compensate for the loss of ERK1. However, ERK has also been reported to *promote* apoptosis in neuronal cells (reviewed by (179)), in fibroblasts (180) as well as in pancreatic beta cells (as discussed below), indicating that the role of ERK in cell survival is not as absolute as previously proposed. It has been proposed that the duration and/or amplitude of ERK signals may affect the decision of cell fate (179; 180). In addition, it has been reported that the protective effects of ERK2 against distinct apoptogenic stimuli are dependent on its cellular localization (181).

Several reports have described a role for the ERK pathway in providing protection against anoikis (70; 163; 176; 182; 183), (reviewed by (94)). For example, it has been shown that laminin-5 engagement by α3β1 integrins promotes keratinocyte cell survival through activation of the MAP kinase ERK pathway (70). However, other groups have reported that ERK is not involved in the control of survival of cells in the context of the ECM. For example, it has been reported that ECM-induced survival of MDCK cells involves the PI3K-Akt pathway, but not

the MAP kinase ERK pathway (161) and that laminin 5-induced breast tumour cell survival and anchorage independence is not affected by either PI3K or ERK activities (184). Finally, it has also been proposed that ERK activation can be involved in signaling leading to anoikis (185) but this remains to be confirmed. Therefore, it appears that the involvement of the MAP kinase ERK pathway in ECM-induced cell survival might be cell-type and/or context-dependent.

1.5. Signaling involving FAK, PI3K-Akt and ERK and the pancreatic beta cell

Although FAK plays a central role in several crucial biological functions (such as cell migration and survival, in particular), no study assessing its expression and/or its function in the pancreatic beta cell was reported previous to, or during this thesis project. By contrast, both PI3K-Akt and the MAP kinase ERK pathways had been reported to be involved in the regulation of the function and/or the survival of the pancreatic beta cell, as described below.

1.5.1. PI3K-Akt and the pancreatic beta cell

The expression of the three Akt isoforms Akt1, Akt2 and Akt3 has been reported for the pancreatic islets and for the transformed beta cell lines HIT15, INS-1 and RINm5F (186). Akt expression in islets is weak as compared to adipocytes, and it is prominently expressed in beta cells as compared to non-beta cells (186). Several stimuli have been shown to promote beta cell survival through the PI3K-Akt pathway. These include glucose (at the 10mM-12mM range of concentration) (187), insulin-like growth factor-I (IGF-I) (188; 189), and glucagon-like peptide-1 (GLP-1) (190; 191). Furthermore, constitutive active Akt1 has been shown to protect INS-1 cells against free fatty-acid induced apoptosis (192). However, whether ECM and/or integrins may affect the activity of Akt in the beta cell has not yet been reported.

Several studies describing transgenic mice expressing constitutively active Akt mutants or dominant-negative (kinase-dead) Akt mutants specifically in pancreatic beta cells have been reported. Overexpression of constitutive active Akt1 (i.e. Akt1 lacking the PH domain, and containing an N-terminal *Src*-myristoylation domain) specifically in the pancreatic beta cells (using the rat insulin II promoter) has been reported by two groups, and both made similar observations (193; 194). It was observed that this mutation leads to an increase of islet beta cell mass that is due both to an increase in the number of beta cells and an increase in beta cell size (193; 194). In addition, Bernal-Mizrachi et al. reported an increase in the proliferation of islet cells (194). An increase in the number of beta cells scattered throughout the exocrine pancreas was also observed in the transgenic mice, leading to the suggestion that active Akt1 might induce neogenesis of beta cells (194). Both groups reported an improvement of glucose tolerance, and hyperinsulinemia was observed in the transgenic mice. Furthermore, this mutation does not affect glucose-stimulated insulin secretion, but it induces resistance towards streptozotocin-induced diabetes (193; 194). These observations led to the following conclusions: 1) Akt1 pathway *in vivo* has major effects on the cell size, number and function of beta cells; 2) altered Akt1 activity might lead to development of type 1 and/or type 2 diabetes. Intriguingly, Tuttle et al. observed an *increase* in the rate of basal islet cell apoptosis, but a decrease in apoptosis induced by streptozotocin in the transgenic mice (193). However, two weaknesses in the model used by these two groups can be noted. First, the constitutively active Akt1 mutant with an N-terminal myristoylation domain was used, and the effects of this mutant

might differ from the physiological effects of activated Akt1 *in vivo*. Second, concerns have been raised about the use of the rat insulin promoter (RIP), because it has been reported that this promoter might affect expression of genes in the hypothalamus (195). Bernal-Mizrachi and colleagues have recently reported the generation of transgenic mice overexpressing a kinase-dead mutant of Akt1 (kdAkt1, which serves as a dominant-negative inhibitor of Akt activity), specifically in the pancreatic beta cells (196). The possibly "leaky" RIP promoter was used, however it was verified that there is no aberrant expression of the RIP-kdAkt1 transgene in the hypothalamus. These mice exhibit impaired glucose tolerance and defective insulin secretion, suggesting that Akt1 is essential for normal beta cell function. Intriguingly, islet morphology and susceptibility to apoptosis were reported to not be altered in these mice compared to wild-type mice.

In summary, these different models provide contrasting results. The *in vitro* studies performed on a wide range of cell types, including beta cells, using different tools, including constitutive active and/or dominant-negative Akt mutants, indicate that Akt plays a general pro-survival role in the pancreatic beta cell. However, *in vivo* overexpression of the constitutive active Akt mutant specifically in beta cells was reported to induce an *increase* in basal beta cell apoptosis, while *in vivo* overexpression of the dominant-negative Akt1 mutant specifically in beta cells was reported to not affect islet morphology or beta cell apoptosis. However, quantification of apoptosis *in vivo* can be problematic, due to rapid clearance of apoptotic cells from tissues by macrophages (26), and this may explain the discrepancies observed between the *in vitro* and *in vivo* models. Furthermore, *in vivo* overexpression of dominant-negative and/or of dominant-positive Akt mutants might induce non-specific side effects. Therefore, further *in vivo* studies are mandatory to better define the role of Akt1 in beta cell survival. One potentially interesting model could be beta-cell specific, conditional Akt knock-out mice.

1.5.2. MAP kinase ERK pathway and the pancreatic beta cell

Several stimuli have been shown to activate ERK in the pancreatic beta cell, including cytokines (IL-1β, (197-199)), glucose (200-202), phorbol esters (202), GLP-1 (203; 204) and KCl-induced depolarization (202). However, whether ECM is able to activate the MAP kinase ERK pathway in the pancreatic beta cell has not yet been reported, either before or during this thesis work.

ERK has been involved in the regulation of function (glucose-stimulated insulin secretion) and survival of beta cells, but both of these proposed functions of ERK are controversial. It has been reported by some groups that glucose-induced ERK activity is not involved in glucose-stimulated insulin secretion (200; 205), but that glucose-induced insulin gene transcription is regulated by ERK (201; 206). However, a recent paper has reported that ERK is involved in glucose-stimulated insulin secretion in MIN6 cells by controlling the

phosphorylation of the cytosolic protein synapsin I (207), and it was proposed that different experimental procedures might explain these contrasting results.

The role of the MAP kinase ERK pathway in beta cell survival is also controversial. Indeed, in the context of cytokine-induced ERK activity, ERK has been reported to be involved in IL-1β-induced nitric oxide (NO) synthesis and NO synthase (NOS) mRNA expression (197), as well as in IL-1β-induced apoptosis (199). Furthermore, the ERK inhibitor PD98059 has been reported to prevent glucose-induced IL-1β release and beta cell dysfunction and death (208). On the other hand, it has been reported that inhibition of ERK aggravates IL-1β-induced death of the unusual cell lines AN-ins and AN-glu (198). It has also been reported that inhibition of ERK activity does not alter glucose-induced beta cell survival (187), nor serum or IGF-I-induced cell survival (189). Finally, it has been proposed by a rather superficial study that there could be a correlation between suppressed ERK phosphorylation and diminished islet survival consecutive to islet isolation and purification (209).

In summary, although both the PI3K-Akt and MAP kinase ERK pathways have been involved in signaling leading to increased beta cell survival, their anti-apoptotic role remains to be confirmed (for the PI3K-Akt pathway), or is still controversial (for the ERK pathway). Furthermore, some reports examining their role in pancreatic beta cells remain to be confirmed, due to lack of conclusive results. Finally, the possible effect of ECM on the activity of these pathways in the pancreatic beta cell has not yet been examined.

1.6. ECM and regulation of gene expression

It has been recognized for over twenty years that ligation of integrins to cognate ECM ligand can alter the expression of genes (reviewed by (88)). Integrin-mediated gene expression has been reported to be involved in many biological functions, including cell cycle regulation and control of cell survival (88; 94). It has been proposed by several groups that integrins can promote cell survival through induction of the expression of anti-apoptotic proteins, including Bcl-2 (71; 210) and the caspase-8 inhibitor c-FLIP (62). Furthermore, overexpression of FAK in a human leukaemia cell line activates a PI3K-Akt pathway leading to increased expression of the Inhibitor of Apoptosis protein (IAP) and improved cell survival (125). Several signaling proteins have been involved in integrin-induced gene expression leading to increased cell survival, and these include FAK, PI3K and Akt (210) the MAPKKK c-Raf-1 (62) and ERK (62; 211). Therefore, it can be concluded that activation of signaling pathways (including PI3K-Akt and ERK pathways) by engaged integrins can induce altered gene expression that ultimately leads to increased cell survival.

2. Specific aims of Paper 1

As described above, ligation of integrins to cognate ECM ligand induces phosphorylation of FAK on residue Tyr-397. Two major pathways have been shown to be able to mediate the pro-survival signaling of activated integrins: the PI3K-Akt and the MAP kinase ERK pathways. Although FAK is not always involved in the activation of these signaling pathways by engaged integrins, the appearance of phosphorylated Tyr-397 residue is considered as a hallmark of integrin signaling.

In the context of the pancreatic beta cell, the signaling pathways activated by engagement of integrins to cognate ECM ligand were completely unexplored at the beginning of this thesis project (i.e. 2001). Furthermore, the expression and/or function of FAK in the beta cell were unknown at that time as well. By contrast, several breakthrough papers investigating the roles of PI3K, Akt and of the MAP kinase ERK in the function and/or survival of the pancreatic beta cell have been reported during that period. Although the pro-survival function of the PI3K-Akt pathway in beta cells is not completely established and that of the ERK pathway is controversial, these pathways are interesting potential candidates for a survival signaling emanating from the ECM leading to improved beta cell survival. As described previously (Part I), we chose to study the effect of 804G-ECM on survival of primary rat pancreatic beta cell as a model for the study of ECM-induced pro-survival signaling pathways that occurs in pancreatic beta cells. In this context, the specific aims were to:

- establish whether 804G-ECM improves survival of pancreatic beta cells in culture

- investigate whether 804G-ECM induces phosphorylation of FAK (on residue Tyr-397), in order to establish whether the 804G-ECM activates integrins expressed on beta cells

- investigate whether 804G-ECM activates pathways involving the signaling kinases Akt and/or ERK

- study the involvement of the PI3K-Akt and MAP kinase ERK pathways in the pro-survival effect of the 804G-ECM

- obtain a preliminary insight into whether the 804G-ECM is able to induce expression of specific genes that might be involved in its pro-survival effects.

3. Paper 1

“ Extracellular Matrix Protects Pancreatic Beta-Cells Against Apoptosis : Role of Short- and Long-Term Signaling Pathways ”

Hammar et al. Diabetes, 53: 2034-2041 (2004)

Extracellular Matrix Protects Pancreatic β-Cells Against Apoptosis

Role of Short- and Long-Term Signaling Pathways

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We have shown previously that culture of β-cells on matrix derived from 804G cells and rich in laminin-5 improves their function. The purpose of this study was to investigate whether this matrix protects β-cells against apoptosis and to elucidate signaling pathways involved. Matrix protected sorted rat β-cells against apoptosis under standard conditions (11.2 mmol/l glucose, 10% serum), after serum deprivation (1% serum), and in response to interleukin-1β (IL-1β; 2 ng/ml), compared with control (poly-L-lysine [pLL]). Caspase-8 activity was reduced in cells cultured on matrix, whereas focal adhesion kinase (FAK), protein kinase B (PKB, or Akt), and extracellular signal–regulated kinase (ERK) phosphorylation was augmented. Treatment (4 h) with an anti-β1 integrin antibody, with the ERK pathway inhibitor PD98059, and/or with the phosphatidylinositol 3-kinase inhibitor LY294002 augmented cell death on 804G matrix but not on pLL. In long-term assays (48 h), PD98059 but not LY294002 drastically augmented cell death on 804G matrix but did so to a lesser extent on pLL. The protein inhibitor of nuclear factor-κB (IκBα) was overexpressed in cells cultured 18 h on matrix with partial blockade by PD98059. In summary, this study provides evidence for activation of signaling pathways and gene expression by extracellular matrix leading to improved β-cell survival. *Diabetes* 53:2034–2041, 2004

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ECM, extracellular matrix; ELISA, enzyme-linked immunosorbent assay; ERK, extracellular signal–regulated kinase; FAK, focal adhesion kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HRP, horseradish peroxidase; IκBα, inhibitor of nuclear factor κB; IL-1β, interleukin-1β; MAP, mitogen-activated protein; MEK1, MAP kinase/ERK kinase; NF-κB, nuclear factor-κB; PI, phosphatidylinositol; PKB, protein kinase B; pLL, poly-L-lysine; TUNEL, terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling.

Epithelial cells deprived of matrix attachment undergo programmed cell death, a form of apoptosis termed anoikis (1). The role of cell anchorage in cell survival has been demonstrated in several cell types (reviewed in 2), including pancreatic islet cells (3). Disengagement from extracellular matrix (ECM) is known to prejudice islet cell survival, notably by inducing apoptosis and necrosis (4). It has been reported that reestablishment of appropriate cell-matrix contacts reduces cell death (3,5); however, the molecular basis for such pro-survival signaling remains largely unknown. Recent evidence implicates integrins in mediating pro-survival signals emanating from the ECM (reviewed in 6). Several signal transduction components activated by integrins, including focal adhesion kinase (FAK) (7,8), phosphatidylinositol (PI) 3-kinase (9,10), and the mitogen-activated protein (MAP) kinase/extracellular signal–regulated kinase (ERK) (reviewed in 11) have been implicated in mechanisms underlying anoikis (reviewed in 12). Recently, it has emerged that PI 3-kinase and its downstream effector protein kinase B (PKB, or Akt) play key roles in the regulation of pancreatic β-cell survival (13–17). The role of the MAP kinase ERK cascade in β-cell survival is controversial. Some studies have suggested an antiapoptotic effect of this cascade (18), whereas others claim that it serves as a mediator of cytokine-induced apoptosis (19–21), this claim in itself being controversial (22). Nevertheless, a possible involvement and impact of PKB/Akt and ERK in outside-in signaling from ECM to β-cells remains to be investigated.

ECM produced by the rat bladder carcinoma cell line 804G is rich in laminin-5 (epiligrin) and also contains fibronectin (23–25). This matrix is able to induce attachment and spreading of many epithelial cell types, including pancreatic β-cells (24,26). Furthermore, our group has shown that this matrix improves β-cell function (27). In the present study, we examined whether the 804G matrix could rescue primary pancreatic β-cells from apoptosis, and we investigated the intracellular pathways involved.

RESEARCH DESIGN AND METHODS

Reagents and antibodies. The following materials were used: PD98059 and LY294002 (Calbiochem, Darmstadt, Germany); In Situ Cell Death Detection Kit (POD; Roche Molecular Biochemicals, Rotkreuz, Switzerland) for terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling

(TUNEL) assay with substrate 3,3′-diaminobenzidine (DAB) from Dako (Carpinteria, CA); cell death detection ELISA^{PLUS} kit (Roche); CaspaTag caspase-8 (LETD) activity kit (Intergen, Oxford, U.K.); Hoechst 33342 (Sigma Fluka, Buchs, Switzerland); propidium iodide (Intergen, Purchase, NY); recombinant rat interleukin-1β (IL-1β; RnD Systems, Abingdon, U.K.) hamster anti-rat CD29 (integrin β1 chain) and hamster IgM control antibodies (Becton Dickinson Biosciences, San Jose, CA); polyclonal anti-phospho-FAK (Tyr-397) and anti-FAK (Biosource International, Camarillo, CA); polyclonal anti-phospho-ERK1/2 (Thr-202/Tyr-204), anti-ERK1/2, anti–phospho-PKB/Akt (Ser-473) and anti-PKB/Akt (Cell Signaling Technology-Bioconcept, Allschwil, Switzerland); monoclonal anti-actin (Chemicon International, Temecula, CA); C-21 anti–inhibitor of nuclear factor (NF)-κBα (IκBα; Santa Cruz Biotechnology, Santa Cruz, CA); anti-mouse horseradish peroxidase (HRP) and anti-rabbit HRP (Amersham Pharmacia Biotech, Dübendorf, Switzerland).

Islet isolation and β-cell purification. All experiments were performed on primary pancreatic β-cells sorted from adult rat islet cells by autofluorescence-activated flow cytometry. Islets of Langerhans were isolated by collagenase digestion of pancreas from male Wistar rats (weighing 150–200 g), followed by Ficoll purification (28). Islets were digested with trypsin and sorted by flow cytometry to obtain β-cells as described (28). This purification procedure yields a population consisting of >95% β-cells (27).

Cell culture. Sorted β-cells were washed and incubated overnight in suspension for recovery as previously described (27). Cells were then resuspended at 4×10^5 cells/ml, and aliquots of 50 μl were plated as droplets on noncoated plastic dishes or on plastic dishes coated with poly-L-lysine (pLL), with 804G matrix, or with laminin-5 purified from 804G (provided by N. Koshikawal and V. Quaranta, San Diego, CA). To assess the effect of the antibody directed against β1 integrin subunit, cells were pretreated for 1 h in suspension with 0.5 μg/ml hamster IgM (control) or with 0.5 μg/ml anti–β1 integrin antibody (Ha2/5) before plating them on pLL- or 804G-coated dishes. When indicated, cells were cultured for 48 h in culture medium containing 1% FCS (serum deprivation). To study the effect of IL-1β, cells were cultured for 48 h in culture medium containing 10% FCS and then treated for 4 h with 2 ng/ml IL-1β. To assess the effects of inhibitors, cells were pretreated for 15 min with 50 μmol/l PD98059 and/or 50 μmol/l LY294002, before plating them on pLL- or 804G-treated dishes. Cells were then cultured in standard medium (10% FCS) for 4 h or for 48 h (fresh inhibitor added at 24 h). DMSO was added to control cells at the same final concentration as that used for the inhibitors. For the protein phosphorylation analysis by Western blot, cells were serum starved for 1–4 h in suspension in serum-free medium containing 0.1% BSA. They were then plated on pLL- or 804G-coated dishes for 30 min in the same medium before performing the protein extraction. For gene expression analysis by low-density gene arrays and by quantitative real-time PCR analysis, cells were serum starved (1% FCS) in suspension for 2 h before plating them on pLL- or 804G-coated dishes (or on noncoated dishes as control for the gene arrays). Cells were then cultured for 18 h in medium containing 1% FCS before mRNA extraction. For the IκBα protein analysis by Western blot, cells were treated as for gene expression analysis and cultured for 24 or 48 h in medium containing 1% FCS before protein extraction. Fresh medium (2 ml) was added every 24 h.

804G matrix preparation. 804G cells (the kind gift of Desmos, San Diego, CA) were grown in Dulbecco's modified Eagle's medium containing 10% FCS and 5.6 mmol/l glucose. Conditioned medium (referred to hereafter as 804G matrix) was prepared as previously described (27).

Coating of plastic dishes with pLL, 804G matrix, and laminin-5. Aliquots (60 μl) of pLL (0.1 mg/ml), crude 804G matrix, or laminin-5 purified from 804G matrix (4 μg/ml in PBS) were layered at the center of 35-mm culture petri dishes (adherent dishes for mammalian cell culture). Dishes were kept in a damp box at 37°C for 18–20 h before being rinsed three times with sterile H_2O and air dried. Uncoated Petri dishes (plastic) or dishes coated with 0.1 mg/ml pLL were used as controls.

Analysis of cell death. To quantify cell death by TUNEL, attached cells were washed with PBS and fixed with 4% paraformaldehyde (20 min at room temperature). After permeabilization with 0.5% Triton X-100 (4 min at room temperature), the TUNEL assay (detecting the free 3-OH strand breaks resulting from DNA degradation) was performed with the In Situ Cell Death Detection Kit, according to the manufacturer's instructions. The preparations were then rinsed with PBS and incubated (10 min at room temperature) with the substrate DAB, and the quantification of dead cells was performed using a light microscope. Unless stated otherwise, results are means ± SD of three independent experiments, with a minimum of 500 β-cells examined for each condition in each experiment. The type of cell death detected by TUNEL was assessed as described by others (29). Cells were stained for 20 min at 37°C with 1 μg/ml Hoechst 33342 (to determine the morphology of the nuclei) and with 1.25 μg/ml propidium iodide (which stains the necrotic nuclei) before fixation and staining with TUNEL reagent. Cells lacking propidium iodide uptake and with condensed nuclei were counted as apoptotic. Late apoptosis (or secondary necrosis) was defined by the uptake of propidium iodide and the presence of nuclear condensation, and necrosis was defined by the uptake of propidium iodide and normal nuclear morphology. Only TUNEL-positive cells were considered for this assessment. The quantification by enzyme-linked immunosorbent assay (ELISA) of mono- and oligonucleosomes present in the cytoplasm of apoptotic cells was performed using the cell death detection ELISA^{PLUS} kit according to the manufacturer's instructions (Roche).

Quantification of caspase-8 activity. To quantify caspase-8 activity, attached cells were treated for 1 h with the fluorescein-labeled peptide FAM-peptide-FMK (which irreversibly binds to active caspase-8) as indicated by the manufacturer (Intergen). At 5 min before the end of the incubation, 25 μmol/l of Hoechst 33342 (final concentration) was added to visualize the nuclei. At the end of the incubation, cells were washed and fixed with 4% paraformaldehyde (20 min at room temperature). The quantification of the number of cells containing active caspase-8 as a percent of total was performed using a fluorescence microscope (Axiocam).

Western blot analysis. To analyze FAK, PKB/Akt, and ERK protein phosphorylation, attached cells were washed with ice-cold PBS without Ca^{+2}/Mg^{+2} supplemented with 1 mmol/l sodium vanadate, and they were lysed in sample buffer 1× (62 mmol/l Tris-Cl, pH6.8, 2% SDS, 5% glycerol, and 1% 2-mercaptoethanol). Protein concentrations were determined with the amido black method (30), and equal amounts of total protein were loaded for SDS-PAGE. To analyze IκBα protein expression, cells were washed with ice-cold PBS and lysed in a buffer containing 150 mmol/l NaCl, 50 mmol/l Tris-HCl, 1% Nonidet P40, 0.25% deoxycholate, 0.1% SDS, 1 mmol/l ditiothreitol, and 1 mmol/l phenylmethylsulfonyl fluoride. After determination of protein concentrations by the method of Lowry, sample buffer 3× was added to the proteins, and the samples were analyzed on an 8% SDS-PAGE gel. All samples, after separation on an SDS-PAGE gel, were electroblotted onto nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany) for immunoblotting with the appropriate antibody. An enhanced chemiluminescence protein detection kit (Amersham Biosciences) and a Kodak image station were used for visualization of the bands.

Gene expression analysis with microarrays. Low-density membrane cDNA microarrays (Mouse Cancer Pathway Finder GEArray; SuperArray, Bethesda, MD) were used to compare gene expression between cells cultured on plastic and those cultured on 804G matrix. After cell culture, RNA from attached cells was isolated using the QIAshredder and RNeasy Mini Kit (Qiagen, Basel, Switzerland) according to the manufacturer's instructions. The RNA quality was verified performing agarose gel electrophoresis, and 1.5 μg of the total RNA (for each condition) was used as the template for the ^{32}P-labeled cDNA probe synthesis performed according to the manufacturer's instructions (SuperArray). The membranes were prehybridized and hybridized with the probe, as indicated by the manufacturer, before exposure to X-ray film for various lengths of time at −80°C.

Quantitative real-time PCR. The results for select genes obtained with the microarrays were verified by real-time PCR. cDNA was synthesized with Superscript II (Invitrogen, Basel, Switzerland) using 1 μg of total RNA in a 20-μl reaction volume. For real-time PCR, the cDNA was amplified using a GeneAmp 5700 sequence detection system (PE Applied Biosystems, Foster City, CA). For this purpose, primers were designed according to Primer Express software (PE Applied Biosystems). The following oligonucleotide primer sets were used: IκBα forward: 5′ TGCTGAGGCACTTCTGAAAGC 3′; IκBα reverse: 5′ TCCTCGAAA GTCTCGGAGGTC 3′. The dsDNA-specific dye SYBR Green I was incorporated into the PCR buffer (PE Applied Biosystems) to allow for quantitative detection of the PCR product. The results were analyzed using ABI Prism 7000 SDS software (PE Applied Biosystems). The house-keeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as internal control.

RESULTS

Effect of 804G matrix on β-cell apoptosis induced by serum deprivation or IL-1β. To determine whether the 804G matrix is able to serve as a survival ligand for primary rat pancreatic β-cells, TUNEL and ELISA were performed on cells cultured on 804G matrix or on pLL (a nonspecific adhesive polymer)-coated Petri dishes. β-Cells were cultured for 48 h on pLL-coated (control) or 804G matrix-coated dishes (hereafter called pLL or 804G, respectively) in medium containing either 10% (control conditions) or 1% FCS (low-serum conditions). The number of apoptotic cells as a percent of the total was

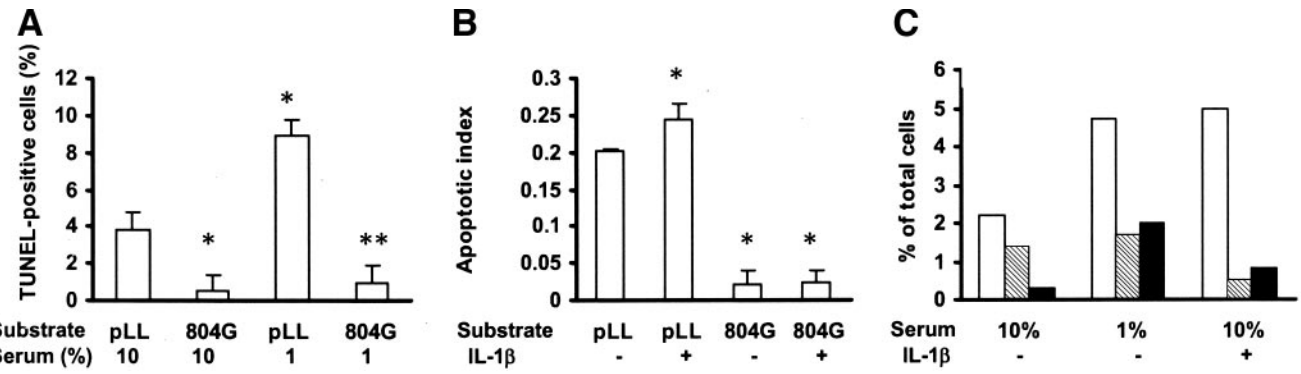

FIG. 1. 804G matrix protects primary pancreatic β-cells from apoptosis. *A*: Cells were cultured for 48 h in medium containing either 10 or 1% FCS before identification of apoptotic (plus necrotic) cells by TUNEL. The number of TUNEL-positive cells as a percent of total was evaluated by optical microscopy. **P* < 0.02 compared with pLL 10% serum; ***P* < 0.001 compared with pLL 1% serum. *B*: After 48 h of culture, cells were treated or not treated with 2 ng/ml IL-1β for 4 h. Apoptosis was measured by ELISA, and apoptotic index is the absorbance [$A_{405\ nm} - A_{490\ nm}$]. Results are means ± SD for *n* = 5 replicates from three independent experiments. **P* < 0.003 relative to pLL (control). *C*: After 48 h of culture and treatment as described above, cells cultured on pLL were stained for 20 min with 1 mg/ml Hoechst 33342 and 1.25 mg/ml propidium iodide for 20 min at 37°C, before fixation and TUNEL analysis. Death was analyzed as described in RESEARCH DESIGN AND METHODS. □, apoptosis; ▨, secondary necrosis; ■ necrosis.

determined under each condition by TUNEL (Fig. 1*A*). Approximately 4% of β-cells cultured on pLL were apoptotic under control conditions. Similar results were obtained when noncoated plastic tissue culture dishes were used instead of pLL-coated dishes (not shown). Culture under low-serum conditions significantly increased the number of TUNEL-positive cells on pLL (9%). When cells were cultured on 804G, <1% of cells were TUNEL positive under control conditions, and serum deprivation did not significantly affect this number.

We next investigated whether the 804G matrix protects β-cells from apoptosis induced by IL-1β, a proinflammatory cytokine that has been shown to be involved in pancreatic β-cell apoptosis leading to the development of type 1 and type 2 diabetes (31,32). For this purpose, the cells were cultured for 48 h on pLL or 804G before being treated for 4 h with 2 ng/ml IL-1β. The apoptosis of β-cells under each condition was measured by ELISA. There was a slight but highly reproducible increase of apoptosis in cells cultured on pLL treated with IL-1β, as compared with nontreated cells ($P < 0.003$). When cells were cultured on 804G, the apoptotic index was significantly decreased as compared with cells cultured on pLL, and there was no difference between nontreated and IL-1β–treated cells (Fig. 1*B*), indicating that this matrix also protects against IL-1β–induced cell death. These results have been confirmed by TUNEL (not shown).

Differential staining of nuclei of TUNEL-positive cells with propidium iodide and Hoechst 33342 distinguished between apoptotic and necrotic cells on pLL (Fig. 1*C*). Under basal conditions (10% FCS), the majority of TUNEL-positive cells were apoptotic or late apoptotic (secondary necrotic). Serum deprivation induced apoptosis and, to a lesser extent, necrosis, whereas IL-1β induced only apoptosis (Fig. 1*C*).

To begin to elucidate the pathway leading to apoptosis on pLL, the activity of caspase-8 in cells cultured for 4 or 48 h on pLL or on 804G was analyzed (Table 1). Caspase-8 activity was higher in cells on pLL compared with those on 804G, and this difference increased with time. These results suggest that the 804G matrix might block caspase-8 activity in turn, explaining the pro-survival effect of the 804G matrix.

Effect of purified laminin-5 on rat islet β-cell survival and spreading. The 804G matrix is known to be rich in laminin-5. However, it also contains fibronectin and may also contain other signaling molecules. To investigate whether laminin-5 is the extracellular molecule responsible for the effect of the 804G matrix, we compared the effects on both cell survival and cell spreading of purified laminin-5 and 804G matrix after 48 h of culture under standard conditions (Figs. 2*A* and *B*). As shown before, there was a significant difference in the number of TUNEL-positive cells between pLL (11%) and 804G (0.5%). The number of TUNEL-positive cells on pure laminin-5 was similar to 804G matrix (Fig. 2*A*). It has been reported that β-cells rapidly flatten and spread on 804G matrix as on pure laminin-5, indicating a strong adhesion of β-cells on these two matrices (27). Here we show that the morphology of β-cells after 48 h of culture on 804G is highly similar to that of cells cultured on pure laminin-5 (Fig. 2*B*). By contrast, rat β-cells do not adhere well and/or do not spread on vitronectin and fibronectin (unpublished results). These results suggest that laminin-5 is the major component of 804G matrix responsible for its pro-survival effect. Therefore, we decided to pursue our studies with the 804G matrix, given its ready availability and convenience of use.

The β1 integrin subunit is involved in the effects of 804G matrix on β-cell survival and spreading. We have previously reported that the laminin-5 receptors integrins α3β1 and α6β1 are expressed on rat β-cells (27,33), and that the latter is involved in the spreading of cells on the 804G matrix (27). To assess whether the β1 integrin subunit is involved in the pro-survival effect of the matrix, we

TABLE 1
Active caspase-8–positive cells

Time	pLL	804G
4 h	2.6 ± 0.9	0.8 ± 0.4*
48 h	4.1 ± 0.5	0.5 ± 0.1†

Data are means ± SD of at least three independent experiments. Rat β-cells were plated on pLL or 804G, and caspase-8 activity was measured after 4 and 48 h. **P* < 0.005 compared with pLL (4 h); †*P* < 0.0002 compared with pLL (48 h).

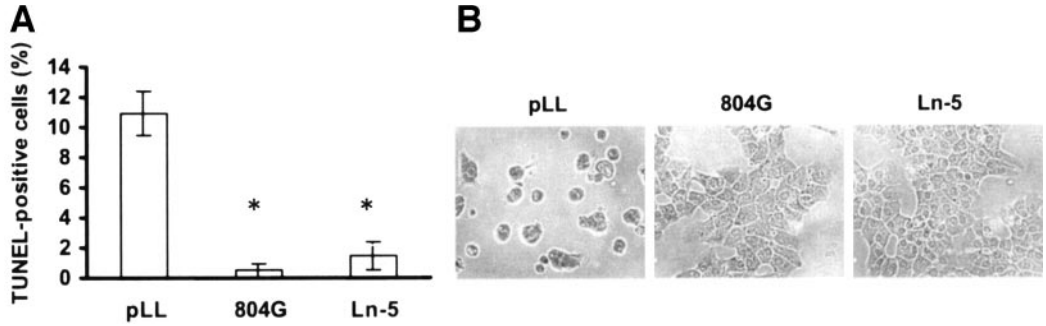

FIG. 2. The spreading and survival of β-cells on purified laminin-5 is similar to that of cells cultured on 804G matrix. *A*: Rat β-cells were cultured for 48 h under standard conditions on pLL-, 804G matrix– or purified laminin-5 (Ln-5)-coated dishes. Apoptotic (plus necrotic cells) were identified by TUNEL. *$P < 0.001$ relative to pLL (control). *B*: Phase-contrast microscopy after 48 h of culture as in panel *A*.

tested the effect of an anti-β1 antibody (Ha2/5) that blocks the adhesion of this integrin to its ECM on the survival of β-cells after 4 h of culture (Fig. 3*A*). On pLL, there was no difference between cells treated with the control antibody IgM and cells treated with Ha2/5. However, on the 804G matrix, the number of TUNEL-positive cells was significantly higher when treated with Ha2/5 (4.9%) compared with control cells (2.3%). Furthermore, the spreading of cells after 4 h of exposure to the 804G matrix was reduced by Ha2/5 (Fig. 3*B*). The control antibody had no effect on cell spreading and cell death at the concentration used (not shown). These results suggest that the β1 integrin subunit mediates the signal from the 804G matrix into the cell, leading to increased spreading and survival.

Effect of 804G matrix on phosphorylation of signaling proteins FAK, PKB/Akt, and ERK. FAK, which is activated by phosphorylation upon integrin ligand binding, mediates survival signaling downstream of integrins and suppresses anoikis and serum withdrawal–induced apoptosis (7,34,35). The PI 3-kinase–PKB/Akt pathway is known to block various apoptotic stimuli. We therefore investigated whether 804G matrix induces activation of FAK and/or PKB/Akt. Cells were serum-starved in suspension for 1 h to reduce constitutive (serum-induced) phosphorylation before plating them for 30 min on pLL or 804G in serum-free medium. Phosphorylation of Tyr397-FAK and Ser473-PKB/Akt was analyzed by Western blot. Adhesion of cells to 804G induced an increased phosphorylation of both proteins compared with adhesion to pLL, whereas the total amount of actin was the same on both substrates (Figs. 4*A* and *B*). These results suggest that this matrix does indeed activate integrin-binding signaling pathways. The amounts of total FAK and total PKB/Akt were slightly decreased in cells on pLL compared with those on 804G (Figs. 4*A* and *B*). It has been reported that caspases cleave specific signaling proteins (including FAK and PKB/Akt) during apoptosis (36). Therefore, the slight decrease of total FAK and PKB/Akt protein amounts in cells cultured on pLL could be due to augmented caspase activity on this substrate compared with matrix.

The FAK signaling complex is known to mediate the activation of the MAP kinase ERK pathway (37,38), a well-known pro-survival pathway in a number of cell types (11). Exposure of serum-starved cells to 804G matrix for 30 min induced higher phosphorylation of ERK1 and ERK2 (Thr-202/Tyr-204 phosphorylation) than pLL (Fig. 4*C*). Attachment of cells to pLL, however, induced higher phosphorylation of ERK proteins as compared with cells in suspension (Fig. 4*C*). In summary, the 804G matrix activates FAK (hallmark of integrin activation) as well as PKB/Akt and ERK pathways.

Signaling pathways involved in pro-survival effects of the 804G matrix. To investigate whether PI 3-kinase–PKB/Akt and ERK pathways are involved in the antiapoptotic effect of 804G matrix, we examined the effects of LY294002 and PD98059. These are specific inhibitors of both PI 3-kinase and MAP kinase/ERK kinase (MEK1), respectively, and were found to be effective in rat β-cells at the concentration used (data not shown). In the short term (4 h), blocking PI 3-kinase and MEK1 activities signifi-

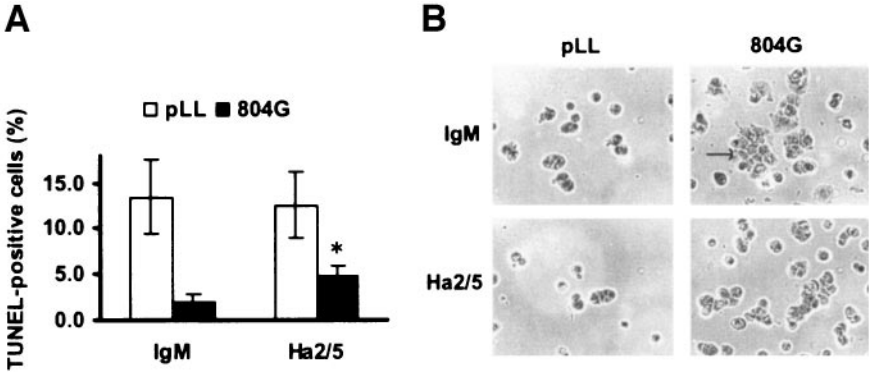

FIG. 3. The β1 integrin subunit is involved in the spreading and improved survival of β-cells induced by the 804G matrix. *A*: Cells were pretreated with control antibody (IgM) or with anti-β1 integrin antibody (Ha2/5) and then attached on pLL- or on 804G-coated dishes. After 4 h of culture under standard conditions, cells were fixed, and cell death was analyzed by TUNEL. *$P < 0.03$ relative to 804G (IgM), pLL (IgM), and pLL (Ha2/5). *B*: Phase-contrast microscopy after 4 h of culture as in panel *A*. A group of spread cells is shown by the arrow.

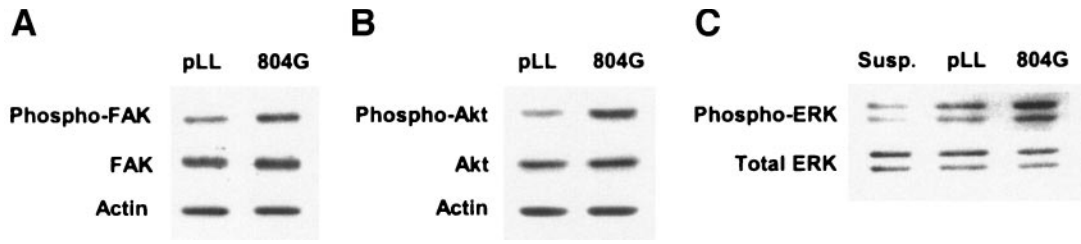

FIG. 4. 804G matrix induces phosphorylation of signaling proteins FAK, PKB/Akt, and ERK. FAK (*A*), PKB/Akt (*B*), and ERK1/2 (*C*) phosphorylation was determined by Western blotting with specific anti-phospho antibodies compared with blotting with antibodies specific for each corresponding protein. Each figure is representative of three independent experiments. Susp., suspension.

cantly augmented cell death on 804G to the same extent (Fig. 5*A*). This treatment did not affect cell death on pLL. The combination of both inhibitors did not alter the effect of each individual inhibitor, suggesting that they block a common pathway activated by the matrix. However, cells on 804G survived better than cells on pLL, even in presence of inhibitors, suggesting that both pathways are only partially involved in the short-term antiapoptotic effect of 804G, or that the inhibitors were only partially effective under these conditions. Blockade of the MAP kinase ERK pathway with PD98059 during 48 h induced a striking increase of the number of apoptotic cells on 804G and also affected cell survival on pLL, albeit to a lesser extent (Fig. 5*B*). In the presence of this inhibitor, attachment to the 804G matrix no longer exerted any protective effect compared with control (pLL). The Western blot analysis (Fig. 4*C*) showed that the attachment on pLL itself induces ERK phosphorylation to a limited extent as compared with suspended cells, although to a lesser extent than on 804G, which may explain why 48 h of treatment with PD98059 also induced higher cell death in cells on pLL. It was also noted that 48 h of culture with PD98059 did not affect the well-spread morphology of the cells cultured on 804G, and that DMSO (used for the dilution of inhibitors) alone had no effect on cell survival and cell spreading at the concentration used (not shown). Treatment of cells with the PI 3-kinase inhibitor LY294002 did not significantly affect cell death on either pLL or 804G after 48 h (Fig. 5*B*), and combined treatment with both inhibitors gave similar results as treatment with PD98059 alone (Fig. 5*B*). These results suggest that the MAP kinase ERK pathway is the dominant one for long-term survival of primary pancreatic β-cells induced by the ECM.

Effect of 804G matrix on gene expression. Differential expression on 804G matrix of genes involved in specific signal transduction pathways such as those implicated in apoptosis and cell cycle was investigated using low-density cDNA microarray membranes (GEArray). Only one gene present on this array was significantly overexpressed in cells cultured for 18 h on the 804G matrix compared with control cells: IκBα (not shown). IκBα has been reported to have an antiapoptotic function in pancreatic β-cells (39). This overexpression was confirmed by real-time quantitative PCR: the IκBα mRNA (normalized to GAPDH mRNA levels) was at least three times more highly expressed in cells cultured on 804G matrix compared with cells cultured on pLL (Fig. 6*A*). IκBα protein levels were measured by Western blot. IκBα protein was increased slightly on 804G vs. pLL after 24 h of culture, with a clear increase by 48 h (Fig. 6*B*). There were no differences in IκBα expression (mRNA and protein) between noncoated and pLL-coated dishes (data not shown). Although this augmented protein expression correlates with the increased gene transcription, we cannot exclude that this difference might be a consequence (partly or completely) of an enhanced degradation of this protein in cells cultured on pLL-coated dishes compared with 804G.

The MAP kinase ERK pathway is involved in IκBα overexpression induced by the 804G matrix. Given that the 804G matrix stimulates the ERK cascade and that this signaling pathway is involved in the pro-survival effects of this matrix, we hypothesized that this cascade might mediate the effect of the matrix on IκBα gene expression. This was tested using the inhibitor PD98059. Treatment of cells with PD98059 did not significantly affect IκBα expression (mRNA) in cells cultured on pLL, but it did reduce by ~60% the IκBα overexpression induced by the 804G matrix (Fig. 6*A*). Thus, these quantitative PCR

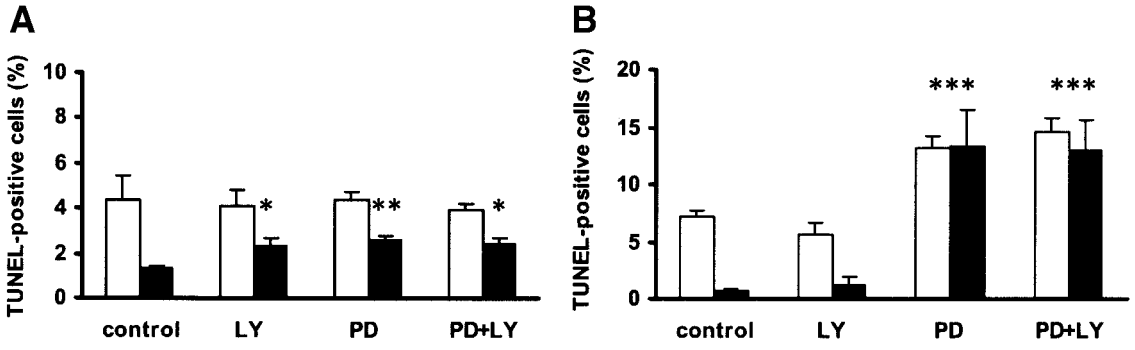

FIG. 5. Involvement of both ERK and Akt/PKB pathways in the pro-survival effect of the matrix. Cells were pretreated with inhibitors of PI 3-kinase (LY294002: LY) and/or of MEK1 (PD98059: PD) and then attached on pLL- or 804G-coated dishes. After 4 h (*A*) or 48 h (*B*), cell death was analyzed by TUNEL. Results are shown as means ± SEM of a minimum of three independent experiments. **P* < 0.04 compared with 804G control; ***P* < 0.01 compared with 804G control; ****P* < 0.005 compared with control conditions (for pLL and 804G). □ pLL; ■, 804G.

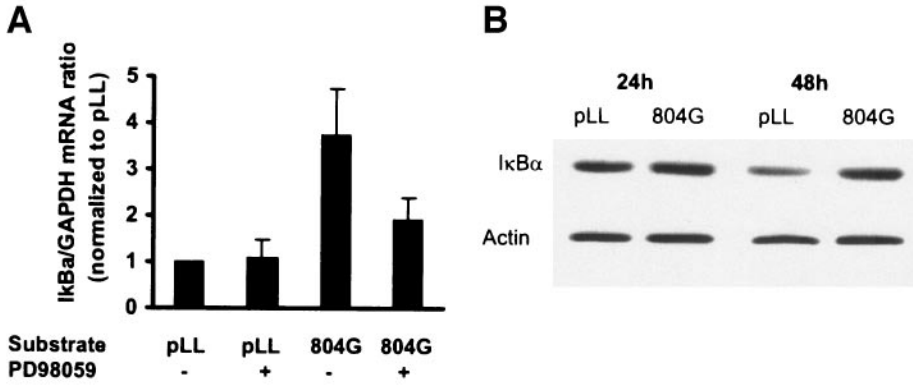

FIG. 6. IκBα expression is induced by the 804G matrix, and this overexpression is reduced by treatment with the ERK inhibitor PD98059. *A*: Cells were treated or not treated with PD98059 before plating them on pLL- versus 804G-coated dishes. After 18 h, RNA was extracted, and real-time PCR was performed. The results (means ± SD from two independent experiments) show the IκBα/GAPDH relative mRNA expression, which was normalized to the control (pLL). *B*: The IκBα protein levels in cells cultured for 24 and 48 h, on pLL or on 804G, were analyzed by Western blotting. This Western blot is representative of three independent experiments.

analyses show that blocking the ERK pathway significantly reduces the effect of the 804G matrix on IκBα gene expression. This result supports the notion that the activation of the MAP kinase ERK cascade by the 804G matrix might contribute to long-term survival of pancreatic β-cells by inducing the expression of antiapoptotic genes.

DISCUSSION

We examined whether ECM can rescue primary pancreatic β-cells from apoptosis and investigated the intracellular pathways involved. The 804G matrix protects pancreatic β-cells from apoptosis under three different conditions: "standard" culture conditions, after serum deprivation, and in response to the cytokine IL-1β. These results demonstrate the importance of adequate ECM for β-cell survival. It is interesting to note that signaling pathways induced by IL-1β in islet β-cells differ when this cytokine is administered in vivo compared with in vitro treatment (40). It has been suggested that one primary difference between the in vivo and the in vitro model systems might be the absence of ECM in the latter (41).

It has been shown that laminin is a component of the basement membrane surrounding islets in vivo, and that the loss of this basement membrane occurring during islet isolation leads to increased cell death (4,42). Experiments performed with laminin-5 purified from 804G matrix (Fig. 2*A* and *B*) suggest that it is the major component of this matrix responsible for its pro-survival effect. The fact that this matrix induces higher phosphorylation of FAK compared with attachment to pLL (Fig. 4*A*) indicates that it engages and activates integrins. Our group has shown that rat pancreatic β-cells express α6β1 and α3β1 integrins (27,33), which have both been identified as receptors for laminin-5. It was shown previously that function-blocking antibodies directed against the α6 integrin subunit blocked cell spreading induced by 804G matrix (27). Here we show that a blocking antibody directed against the β1 integrin subunit reduces both the spreading and the improved survival induced by the 804G matrix (Fig. 3*A* and *B*). Collectively, these data lead us to suggest that α6β1 integrin is a major conduit for outside-in signaling pathway in β-cells established on this particular matrix.

The intracellular molecule(s) responsible for transducing cell-matrix signaling in β-cells have not been studied previously in detail. We now show that compared with pLL, attachment of cells to the 804G matrix induces higher levels of phosphorylation of both MAP kinase ERK and PKB/Akt (Figs. 4*B* and *C*). Furthermore, apoptosis of cells cultured on 804G was increased in the presence of the MEK1 inhibitor PD98059 (4 and 48 h) and/or in the presence of the PI 3-kinase inhibitor LY294002 (4 h only) (Figs. 5*A* and *B*). These results strongly suggest that the short-term pro-survival effect emanating from the 804G matrix is mediated by both of these pathways, whereas longer term effects are mediated at least in part by MAP kinase ERK but not by PKB/Akt.

We propose that the role of both the PI 3-kinase–PKB/Akt and MAP kinase ERK pathways in mediating the short-term (4 h) pro-survival effects of the 804G matrix may be related to their effects on preventing the cleavage and subsequent activation of procaspase-8. Indeed, it has been reported that activated PKB/Akt prevents apoptosis by inhibiting death-inducing signaling complex assembly, thus preventing procaspase-8 cleavage (43), whereas MAP kinase activation deflects DISC signaling from activating caspase-8 (44). This would also explain the nonadditive effect of both inhibitors on short-term cell survival. During the short-term studies, cells on 804G matrix survived better than cells on pLL, even in the presence of inhibitors, suggesting that the ERK and PKB/Akt pathways may be only two components of the short-term antiapoptotic effect of the matrix. A potential mechanism contributing to cell death on pLL, presumably unrelated to ERK and PKB/Akt pathways, is "integrin-mediated death," whereby unligated integrins in cells adhering to substrates devoid of appropriate ECM ligands can act as negative regulators of cell survival (45,46). Unligated integrins are reported to promote apoptosis by recruitment of caspase-8 to the plasma membrane independent of death receptors or Fas-associating protein with a death domain. Our results showing that caspase-8 activation is significantly reduced in cells cultured on 804G compared with cells cultured on pLL (Table 1) support both of the above hypotheses.

The present work shows for the first time the importance of the ERK pathway in promoting survival of pancreatic β-cells induced by the ECM. The fact that the ERK

pathway is involved in the antiapoptotic effect of the 804G matrix on pancreatic β-cells is intriguing because activation of this pathway has been reported to be involved in cytokine-induced apoptosis in β-cells (19,47). One potential explanation for this discrepancy is that cytokine- and ECM-induced activation of ERKs may be significantly different in terms of duration and intensity, which may lead to opposite effects on life-or-death decisions by the cell (48).

The finding that the 804G matrix induces overexpression of IκBα is most interesting because IκBα has been shown to inhibit NF-κB nuclear translocation and transcriptional activity (49). In general, NF-κB is reported to mediate antiapoptotic signals. However, NF-κB is considered as an important transcription factor, mediating IL-1β–induced signal transduction and regulating groups of genes contributing to death in pancreatic β-cells (31). Overexpression of a nondegradable IκB mutant, which specifically blocks cytokine-induced NF-κB activation, prevents β-cell apoptosis (39). The overexpression of the IκBα gene induced by the 804G matrix may thus contribute to its antiapoptotic effect. It is well established that ECM can influence gene expression via the MAP kinase ERK pathway (11). In the present work, we show that activation of the ERK pathway by the 804G matrix mediates the overexpression of IκBα in β-cells. This suggests that activation of the MAP kinase ERK cascade by the 804G matrix might contribute to long-term survival of pancreatic β-cells by inducing the expression of antiapoptotic genes.

Inadequate culture conditions have been suggested to be responsible, at least in part, for the important loss of islet cell viability before transplantation in diabetic patients. A prerequisite for the improvement of islet cell maintenance in vitro, and consequently for increased survival of the grafted cells, is to gain better knowledge of both the intracellular pathways involved in cell survival and factors regulating these signaling pathways. In this context, these results are particularly relevant in providing an insight into important signaling pathways activated by ECM, which lead to improved pancreatic β-cell survival before transplantation.

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REFERENCES

1. Frisch SM, Francis H: Disruption of epithelial cell-matrix interactions induces apoptosis. *J Cell Biol* 124:619–626, 1994
2. Grossmann J: Molecular mechanisms of "detachment-induced apoptosis—Anoikis." *Apoptosis* 7:247–260, 2002
3. Wang RN, Rosenberg L: Maintenance of beta-cell function and survival following islet isolation requires reestablishment of the islet-matrix relationship. *J Endocrinol* 163:181–190, 1999
4. Rosenberg L, Wang R, Paraskevas S, Maysinger D: Structural and functional changes resulting from islet isolation lead to islet cell death. *Surgery* 126:393–398, 1999
5. Ris F, Hammar E, Bosco D, Pilloud C, Maedler K, Donath MY, Oberholzer J, Zeender E, Morel P, Rouiller D, Halban PA: Impact of integrin-matrix matching and inhibition of apoptosis on the survival of purified human beta-cells in vitro. *Diabetologia* 45:841–850, 2002
6. Miranti CK, Brugge JS: Sensing the environment: a historical perspective on integrin signal transduction. *Nat Cell Biol* 4:E83–E90, 2002
7. Frisch SM, Vuori K, Ruoslahti E, Chan-Hui PY: Control of adhesion-dependent cell survival by focal adhesion kinase. *J Cell Biol* 134:793–799, 1996
8. Ilic D, Almeida EA, Schlaepfer DD, Dazin P, Aizawa S, Damsky CH: Extracellular matrix survival signals transduced by focal adhesion kinase suppress p53-mediated apoptosis. *J Cell Biol* 143:547–560, 1998
9. Khwaja A, Rodriguez-Viciana P, Wennstrom S, Warne PH, Downward J: Matrix adhesion and Ras transformation both activate a phosphoinositide 3-OH kinase and protein kinase B/Akt cellular survival pathway. *Embo J* 16:2783–2793, 1997
10. Lee JW, Juliano RL: Alpha5beta1 integrin protects intestinal epithelial cells from apoptosis through a phosphatidylinositol 3-kinase and protein kinase B-dependent pathway. *Mol Biol Cell* 11:1973–1987, 2000
11. Howe AK, Aplin AE, Juliano RL: Anchorage-dependent ERK signaling–mechanisms and consequences. *Curr Opin Genet Dev* 12:30–35, 2002
12. Frisch SM, Screaton RA: Anoikis mechanisms. *Curr Opin Cell Biol* 13:555–562, 2001
13. Contreras JL, Smyth CA, Bilbao G, Young CJ, Thompson JA, Eckhoff DE: Simvastatin induces activation of the serine-threonine protein kinase AKT and increases survival of isolated human pancreatic islets. *Transplantation* 74:1063–1069, 2002
14. Liu W, Chin-Chance C, Lee EJ, Lowe WL Jr: Activation of phosphatidylinositol 3-kinase contributes to insulin-like growth factor I-mediated inhibition of pancreatic beta-cell death. *Endocrinology* 143:3802–3812, 2002
15. Srinivasan S, Bernal-Mizrachi E, Ohsugi M, Permutt MA: Glucose promotes pancreatic islet beta-cell survival through a PI 3-kinase/Akt-signaling pathway. *Am J Physiol Endocrinol Metab* 283:E784–E793, 2002
16. Tuttle RL, Gill NS, Pugh W, Lee JP, Koeberlein B, Furth EE, Polonsky KS, Naji A, Birnbaum MJ: Regulation of pancreatic beta-cell growth and survival by the serine/threonine protein kinase Akt1/PKBalpha. *Nat Med* 7:1133–1137, 2001
17. Wrede CE, Dickson LM, Lingohr MK, Briaud I, Rhodes CJ: Protein kinase B/Akt prevents fatty acid-induced apoptosis in pancreatic beta-cells (INS-1). *J Biol Chem* 277:49676–49684, 2002
18. Paraskevas S, Aikin R, Maysinger D, Lakey JR, Cavanagh TJ, Hering B, Wang R, Rosenberg L: Activation and expression of ERK, JNK, and p38 MAP-kinases in isolated islets of Langerhans: implications for cultured islet survival. *FEBS Lett* 455:203–208, 1999
19. Pavlovic D, Andersen NA, Mandrup-Poulsen T, Eizirik DL: Activation of extracellular signal-regulated kinase (ERK)1/2 contributes to cytokine-induced apoptosis in purified rat pancreatic beta-cells. *Eur Cytokine Netw* 11:267–274, 2000
20. Larsen CM, Wadt KA, Juhl LF, Andersen HU, Karlsen AE, Su MS, Seedorf K, Shapiro L, Dinarello CA, Mandrup-Poulsen T: Interleukin-1beta-induced rat pancreatic islet nitric oxide synthesis requires both the p38 and extracellular signal-regulated kinase 1/2 mitogen-activated protein kinases. *J Biol Chem* 273:15294–15300, 1998
21. Mandrup-Poulsen T: β-Cell apoptosis: stimuli and signaling. *Diabetes* 50 (Suppl. 1):S58–S63, 2001
22. Ammendrup A, Maillard A, Nielsen K, Aabenhus Andersen N, Serup P, Dragsbaek Madsen O, Mandrup-Poulsen T, Bonny C: The c-Jun amino-terminal kinase pathway is preferentially activated by interleukin-1 and controls apoptosis in differentiating pancreatic β-cells. *Diabetes* 49:1468–1476, 2000
23. Baker SE, DiPasquale AP, Stock EL, Quaranta V, Fitchmun M, Jones JC: Morphogenetic effects of soluble laminin-5 on cultured epithelial cells and tissue explants. *Exp Cell Res* 228:262–270, 1996
24. Hormia M, Falk-Marzillier J, Plopper G, Tamura RN, Jones JC, Quaranta V: Rapid spreading and mature hemidesmosome formation in HaCaT keratinocytes induced by incubation with soluble laminin-5r. *J Invest Dermatol* 105:557–561, 1995
25. Langhofer M, Hopkinson SB, Jones JC: The matrix secreted by 804G cells contains laminin-related components that participate in hemidesmosome assembly in vitro. *J Cell Sci* 105:753–764, 1993
26. Hayek A, Beattie GM, Cirulli V, Lopez AD, Ricordi C, Rubin JS: Growth factor/matrix-induced proliferation of human adult β-cells. *Diabetes* 44: 1458–1460, 1995
27. Bosco D, Meda P, Halban PA, Rouiller DG: Importance of cell-matrix interactions in rat islet β-cell secretion in vitro: role of α6β1 integrin. *Diabetes* 49:233–243, 2000
28. Rouiller DG, Cirulli V, Halban PA: Differences in aggregation properties

and levels of the neural cell adhesion molecule (NCAM) between islet cell types. *Exp Cell Res* 191:305–312, 1990
29. Kelly KJ, Sandoval RM, Dunn KW, Molitoris BA, Dagher PC: A novel method to determine specificity and sensitivity of the TUNEL reaction in the quantitation of apoptosis. *Am J Physiol Cell Physiol* 284:C1309–C1318, 2003
30. Schaffner W, Weissmann C: A rapid, sensitive, and specific method for the determination of protein in dilute solution. *Anal Biochem* 56:502–514, 1973
31. Eizirik DL, Mandrup-Poulsen T: A choice of death–the signal-transduction of immune-mediated beta-cell apoptosis. *Diabetologia* 44:2115–2133, 2001
32. Maedler K, Sergeev P, Ris F, Oberholzer J, Joller-Jemelka HI, Spinas GA, Kaiser N, Halban PA, Donath MY: Glucose-induced beta cell production of IL-1beta contributes to glucotoxicity in human pancreatic islets. *J Clin Invest* 110:851–860, 2002
33. Kantengwa S, Baetens D, Sadoul K, Buck CA, Halban PA, Rouiller DG: Identification and characterization of alpha 3 beta 1 integrin on primary and transformed rat islet cells. *Exp Cell Res* 237:394–402, 1997
34. Hungerford JE, Compton MT, Matter ML, Hoffstrom BG, Otey CA: Inhibition of pp125FAK in cultured fibroblasts results in apoptosis. *J Cell Biol* 135:1383–1390, 1996
35. Almeida EA, Ilic D, Han Q, Hauck CR, Jin F, Kawakatsu H, Schlaepfer DD, Damsky CH: Matrix survival signaling: from fibronectin via focal adhesion kinase to c-Jun NH(2)-terminal kinase. *J Cell Biol* 149:741–754, 2000
36. Widmann C, Gibson S, Johnson GL: Caspase-dependent cleavage of signaling proteins during apoptosis: a turn-off mechanism for anti-apoptotic signals. *J Biol Chem* 273:7141–7147, 1998
37. Renshaw MW, Price LS, Schwartz MA: Focal adhesion kinase mediates the integrin signaling requirement for growth factor activation of MAP kinase. *J Cell Biol* 147:611–618, 1999
38. Parsons JT: Focal adhesion kinase: the first ten years. *J Cell Sci* 116:1409–1416, 2003
39. Heimberg H, Heremans Y, Jobin C, Leemans R, Cardozo AK, Darville M, Eizirik DL: Inhibition of cytokine-induced NF-κB activation by adenovirus-mediated expression of a NF-κB super-repressor prevents β-cell apoptosis. *Diabetes* 50:2219–2224, 2001
40. Todaro M, Di Gaudio F, Lavitrano M, Stassi G, Papaccio G: Islet beta-cell apoptosis triggered in vivo by interleukin-1beta is not related to the inducible nitric oxide synthase pathway: evidence for mitochondrial function impairment and lipoperoxidation. *Endocrinology* 144:4264–4271, 2003
41. Lowe WL Jr: "The matrix unloaded": implications for cytokine signaling in islets? *Endocrinology* 144:4262–4263, 2003
42. Wang RN, Paraskevas S, Rosenberg L: Characterization of integrin expression in islets isolated from hamster, canine, porcine, and human pancreas. *J Histochem Cytochem* 47:499–506, 1999
43. Jones RG, Elford AR, Parsons MJ, Wu L, Krawczyk CM, Yeh WC, Hakem R, Rottapel R, Woodgett JR, Ohashi PS: CD28-dependent activation of protein kinase B/Akt blocks Fas-mediated apoptosis by preventing death-inducing signaling complex assembly. *J Exp Med* 196:335–348, 2002
44. Holmstrom TH, Schmitz I, Soderstrom TS, Poukkula M, Johnson VL, Chow SC, Krammer PH, Eriksson JE: MAPK/ERK signaling in activated T cells inhibits CD95/Fas-mediated apoptosis downstream of DISC assembly. *Embo J* 19:5418–5428, 2000
45. Cheresh DA, Stupack DG: Integrin-mediated death: an explanation of the integrin-knockout phenotype? *Nat Med* 8:193–194, 2002
46. Stupack DG, Puente XS, Boutsaboualoy S, Storgard CM, Cheresh DA: Apoptosis of adherent cells by recruitment of caspase-8 to unligated integrins. *J Cell Biol* 155:459–470, 2001
47. Donath MY, Storling J, Maedler K, Mandrup-Poulsen T: Inflammatory mediators and islet beta-cell failure: a link between type 1 and type 2 diabetes. *J Mol Med* 81:455–470, 2003
48. Rul W, Zugasti O, Roux P, Peyssonnaux C, Eychene A, Franke TF, Lenormand P, Fort P, Hibner U: Activation of ERK, controlled by Rac1 and Cdc42 via Akt, is required for anoikis. *Ann N Y Acad Sci* 973:145–148, 2002
49. Dixit V, Mak TW: NF-kappaB signaling: many roads lead to Madrid. *Cell* 111:615–619, 2002

4. Major findings and perspectives of Part II (Paper 1)

In this first part of the thesis work, the aims were the following: 1) to establish whether the interaction of primary rat pancreatic beta cells with an appropriate ECM (804G-ECM) might promote survival and 2) to get a preliminary insight into the pathways involved.

This work establishes that 804G-ECM protects beta cells from death (both apoptosis and necrosis) as compared to control substrate pLL. This was assessed after 48h of culture in three different culture conditions: in presence of 10% FCS, in presence of 1% FCS ("serum-deprived"), as well as in presence of the cytokine IL-1β. Beta cells cultured on 804G-ECM display reduced levels of caspase-8 activity compared to cells on pLL, providing a possible explanation for the anti-apoptotic effect of 804G-ECM. It was also found that the interaction between the ECM protein laminin-5 and the β1 integrin subunit is involved in the pro-survival effect of 804G-ECM. The phosphorylation state of the signaling proteins FAK, Akt and ERK on specific residues (reflecting their activity) was assessed by western blot. It was found that 804G-ECM induces a rapid (within 30 minutes) phosphorylation of all of these kinases. The appearance of phosphorylated FAK sustains the hypothesis that 804G-ECM induces integrin signaling. To investigate the roles of the PI3K-Akt signaling module and the MAP kinase ERK pathway in the pro-survival effect of the 804G-ECM, the effect of blocking these pathways using pharmacological inhibitors was assessed. Inhibition of both of the PI3K-Akt and ERK pathways reduces the short-term pro-survival effect of the 804G-ECM (4h), while only inhibition of the ERK pathway abrogates the long-term (48h) anti-apoptotic effect of the 804G-ECM. Finally, low-density microarrays using cDNA derived from beta cells cultured for 18h on pLL or on 804G-ECM were performed to assess whether 804G-ECM is able to induce expression of genes. One gene was found to be overexpressed in cells cultured on 804G-ECM as compared to cells on pLL: the inhibitor of NF-κB (IκBα). Furthermore, it was found that the MAP kinase ERK pathway is involved in this 804G-ECM-induced overexpression of IκBα.

In summary, these results provide a first insight into signaling pathways induced by the 804G-ECM leading to increased gene expression and improved cell survival. While many papers have reported a pro-survival role for Akt in beta cells, such a role for ERK is more controversial. For example, ERK has been shown to mediate cytokine-induced beta cell death. However, as discussed in Paper 1, cytokine- and 804G-ECM-induced activation of ERKs may significantly differ in terms of duration and intensity, which may lead to opposite effects on the cell. Furthermore, it has been recently reported that subcellular localization of activated ERK also plays a role in determining its biological outcomes (181). Therefore, it should be interesting to compare the subcellular localization, duration and intensity of ERK activity induced by 804G-ECM compared to that induced by the cytokine IL-1β.

What could be the downstream effectors of 804G-ECM-activated ERK and Akt/PKB? To date, over 50 ERK substrates have been characterized, including protein kinases,

transcription factors, transmembrane receptors and structural proteins, among others. These are distributed in different cellular compartments, including cytoplasm, nucleus and cytoskeleton (169). One ERK substrate that is an interesting candidate in the context of diabetes is the cAMP-reponse element binding protein (CREB). Loss of the ERK-CREB signaling pathway has been proposed to be involved in beta cell apoptosis induced by long-term hyperglycemia (212). Furthermore, CREB has been reported to mediate cAMP-mediated signaling leading to increased beta cell survival (213). In addition, mice deficient in CREB activity have been reported to develop diabetes secondary to beta cell apoptosis (213). Akt has a myriad of protein substrates involved in its anti-apoptotic signaling as well. In the context of the pancreatic beta cell survival, the potentially interesting substrates include the pro-apoptotic member of Bcl-2 family Bad, the X-linked inhibitor of apoptosis protein (XIAP), glycogen synthase kinase 3β (GSK3β) and forkhead transcription factors (including FoxO-1) (9; 192).

One unexpected and particularly interesting result was the finding that 804G-ECM induces the overexpression of IκBα. We found it most important to pursue this path not only because IκBα inhibits the transcription factor NF-κB, but also because its expression is *induced* by NF-κB. In addition, NF-κB has been reported to be a downstream effector of both the PI3K-Akt and MAP kinase ERK pathways leading to increased cell survival. This led to the hypothesis that 804G-ECM might induce the transcriptional activity of NF-κB, which is the central hypothesis for the next part (Part III, Paper 2) of this work.

PART III (PAPER 2):

ACTIVATION OF THE TRANSCRIPTION FACTOR NF-κB BY ECM

1. Introduction to Part III (Paper 2)

1.1. General Introduction on NF-κB

The transcription factor NF-κB was discovered in 1986 as a factor in the nucleus of B cells that binds to the enhancer of the kappa light chain of immunoglobulin (214). Since, NF-κB has been shown to be expressed ubiquitously in the cytoplasm of all cell types, and to be conserved from Drosophila to man. NF-κB is a complex signaling module that plays a critical role in many biological processes, in response to various environmental stimuli. NF-κB regulates the expression of cytokines, growth factors and effector enzymes in response to many extra-cellular stimuli (215). The biological system in which the role of NF-κB seems to be the most important is the immune system (216). However, NF-κB has also been reported to play important roles in other biological processes such as embryonic development and the development, and the physiology of tissues such as the mammary gland, bone, skin and central nervous system (215). Dysregulation of NF-κB activity can mediate a wide variety of diseases (217), including cancer (218). In addition, NF-κB has been reported to play a role in the development of diabetes, at both the beta cell and insulin target tissues levels (217; 219). The role of NF-κB in the pancreatic beta cell is described below (Part III, § 1.5).

The mammalian NF-κB family consists of five members: p65 (RelA), RelB, c-Rel, p50/p105 (NF-κB1), and p52/p100 (NF-κB2), (Fig. 8). NF-κB1 and NF-κB2 are synthesized as large precursors, p105 and p100, that are post-translationally processed to the DNA-binding subunits p50 and p52, respectively. NF-κB proteins are characterized by the presence of a conserved 300-amino acid motif Rel homology domain (RHD), containing a nuclear localization sequence (NLS), that is located at the N-terminus and is responsible for dimerization, interaction with inhibitors of NF-κB (IκBs) and sequence-specific binding to DNA. In addition, p65 (RelA), RelB and c-Rel contain transactivation domains (TAD) in their C-terminus. The NF-κB proteins are able to form several different hetero- and/or homo-dimers that differentially control gene expression and are thus associated with specific biological responses. Dimers that contain RelA (p65) or c-Rel are transcriptional activators (215; 216; 220). The most common and studied dimer, which is simply called NF-κB, is the p50/p65 (RelA) heterodimer.

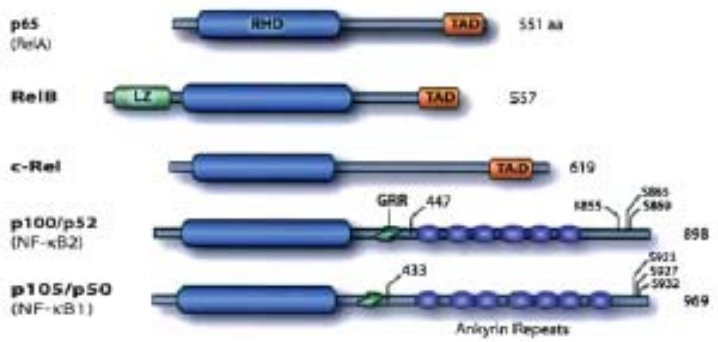

Fig. 8: Schematic representation of the NF-κB family of proteins. RHD: Rel homology domain; TAD: transactivation domain. Reproduced from reference (215).

Two main pathways leading to activation of NF-κB have been defined: the classical ("canonical") pathway and the alternative pathway (Fig. 9). These two pathways are thought to have distinct regulatory functions (216; 220). The canonical pathway is the best-characterized one, while the alternative pathway has been described recently (220). In the canonical pathway, in non-stimulated cells, NF-κB dimers (that can consist either of p50-RelA (p65) or of p52-RelA (p65) complexes) remain in the cytoplasm, where they are bound to and inactivated by inhibitory IκB subunits (221). Upon stimulation of the cell (with the pro-inflammatory cytokine TNFα, for example), signaling pathways lead to activation of the IKK (IκB kinase) complex resulting in phosphorylation of the IκB proteins which are subsequently recognized by the ubiquitin ligase machinery and targeted for degradation (222). The freed NF-κB dimers translocate to the nucleus, where they bind to specific sequences in the promoter or enhancer regions of target genes (215). In the pancreatic beta cell, only the canonical pathway leading to NF-κB activation has been described to date. Therefore, we will from now on focus on this pathway and on the p50/p65 (RelA) heterodimer. In addition to translocation to the nucleus subsequent to IκB degradation, regulation of p65 transcriptional activity also occurs by post-translational modifications, independently of IκB proteins. Indeed, increasing evidence indicates that phosphorylation and degradation of IκB and subsequent liberation of NF-κB are not sufficient to activate NF-κB-dependent transcription. One means of modulating the NF-κB transcriptional activity is by site-specific phosphorylation and acetylation of the p65 subunit itself (223-227).

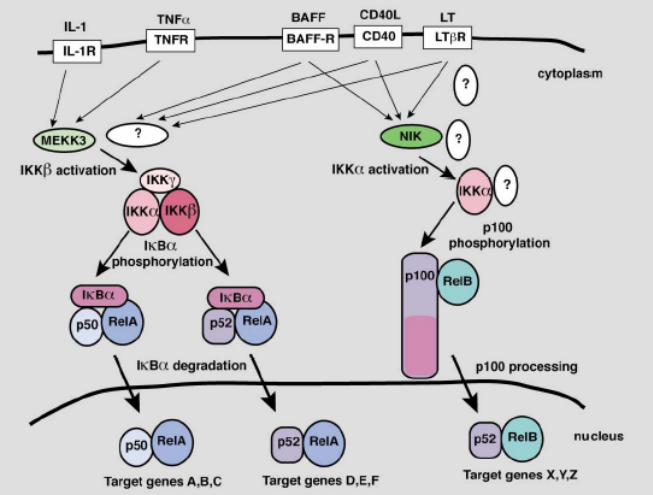

Fig. 9: Two pathways leading to NF-κB activation: canonical (left), and non-canonical (right). Reproduced from reference (220).

Once activated, NF-κB can then be down-regulated by multiple mechanisms. The best characterized pathway involves the IκBα proteins. As the IκBα gene is an NF-κB target, activation of NF-κB leads to generation of newly synthesized IκBα proteins that are able to inhibit nuclear translocation of NF-κB (215; 228-231). In addition, newly synthesized IκBα proteins have been reported to be able to export NF-κB from the nucleus into the cytoplasm (232). Feed-back inhibition of NF-κB activity by the newly synthesized IκBα proteins leads to oscillations and thus temporal control of NF-κB activity. It has been proposed that precise temporal regulation of NF-κB activity by IκBα-induced feed-back inhibition allows an additional level in the control of gene expression (230; 231).

1.2. Functions of NF-κB

NF-κB regulates the expression of genes involved in many different biological processes, such as proliferation, cell spreading and cell motility, differentiation, angiogenesis, normal immune function, and regulation of cell survival (218; 225). As the 804G-ECM has been shown to induce cell spreading (5) and cell survival (Paper 1), we were particularly interested in the possible roles of NF-κB in the two latter biological functions.

1.2.1. Role of NF-κB in cell spreading and cell migration

In the context of cancer, a number of studies have focused on the role of NF-κB in cellular invasion (reviewed by (218)). Cellular invasion involves migration of cells, a process that requires cell anchorage and spreading (233). It has been shown that NF-κB induces expression of several genes that are involved in spreading and/or migration of cells, including chemokines (such as IL-8 and IP-10) and matrix metalloproteinases (MMPs, (218; 234)). Furthermore, NF-κB is involved in Fas apoptosis inhibitory molecule (FAIM)-induced neurite outgrowth (235) and NF-κB-dependent gene expression has been shown to be required for αvβ5-dependent cell motility on ECM (236). Finally, it has been proposed that there might be a functional relationship between the NF-κB subunit p65 and the actin cytoskeleton (237), suggesting that NF-κB may play a yet uncharacterized role in the cytoplasm.

1.2.2. Role of NF-κB in the control of apoptosis

NF-κB has been reported to both play pro-apoptotic and anti-apoptotic roles. The function of NF-κB in cell survival has been proposed to depend on the context of activation, type of inducer and/or cell type (224; 238). The last years have witnessed an explosion in the number of publications focusing on the role of NF-κB in apoptosis (more than 3000 PubMed-cited publications between 1999 and 2004), and this topic will only be briefly summarized here.

The first evidence of the anti-apoptotic role of NF-κB came from the analysis of p65-/- mice that died on embryonic day 15 from massive liver apoptosis (239). Embryonic fibroblasts (EFs) derived from these p65-/- mice are more susceptible to TNFα-induced apoptosis than control EFs, and this can be corrected by p65 reexpression (240). The role of NF-κB in preventing TNFα-induced apoptosis was confirmed the same year by three groups using a mutant, non-phosphorylatable form of the inhibitor of NF-κB (IκB) that acts as “super-repressor” by sequestering NF-κB in the cytoplasm (241-243). Since, NF-κB has been found to protect numerous cell types against diverse apoptotic insults, and to induce expression of many anti-apoptotic genes, such as the inhibitor of apoptosis (IAP) proteins, c-FLIP, or Bcl-2 family proteins (224; 238). Furthermore, NF-κB is also able to repress induction of pro-apoptotic genes

such as Bax (244), and to inhibit transcriptional activity of the pro-apoptotic transcription factor GADD153/CHOP (245). Finally, NF-κB has been shown to play a role in growth factor-induced survival signaling including that induced by PDGF (246).

On the other hand, NF-κB has also been reported to play pro-apoptotic roles (224; 238). It can induce expression of pro-apoptotic genes, such as FasL following T-cell receptor engagement (247; 248), and also repress anti-apoptotic gene expression following ultraviolet C radiation (249). Intriguingly, as described below, the majority of studies investigating the role of NF-κB in pancreatic beta cells have led to the conclusion that it plays a pro-apoptotic role in these cells.

1.3. Activation of NF-κB by ECM

As mentioned in Part II (§ 1.6. ECM and regulation of gene expression), the effect of ECM on the expression of genes is now well acknowledged (88; 250). Lin and colleagues were the first to show that fibronectin attachment specifically increases nuclear translocation and transcriptional activity of the p50/p65 NF-κB heterodimer in monocytic leukemia cells THP-1 (251). Subsequently, activation of NF-κB following attachment to ECM was reported to occur in other cell types, such as fibroblasts, smooth muscle cells (on fibronectin, (252)), and endothelial cells (by EHS matrix (253), or by the αvβ3 ligand osteopontin (254)). The effects of fibronectin on monocyte gene expression has been analyzed by a global gene expression analysis (GeneCalling (255)). This analysis revealed that adhesion of cells to fibronectin results in increased expression of a large number of genes regulated by NF-κB, and that this increased gene expression is strongly potentiated by growth factors (255). However, since cells in suspension (and not attached cells) were used as controls for this gene expression analysis, and since simple attachment of monocytes onto plastic activates NF-κB (256; 257), these latter data remain to be confirmed using a more appropriate control (e.g. attached cells).

Several groups have shown that antibody-induced crosslinking of β1 integrins can induce nuclear translocation and transcriptional activity of NF-κB (175; 211; 251; 258; 259). The global effect of the engagement of α5β1 integrins by fibronectin on endothelial cell (HUVEC) gene expression has been analyzed, using cells plated on pLL as control (260). This study confirmed that α5β1 integrin engagement by fibronectin is sufficient to activate a broad gene expression program, and that endothelial cell attachment activates the NF-κB pathway of gene induction (260). By contrast, adhesion of endothelial cells to laminin through α2β1 integrin has a much more modest effect on NF-κB activity and on gene expression pattern (260). The effect of a given integrin on NF-κB activity appears to be cell-type specific. For example, in endothelial cells, laminin does scarcely affect NF-κB activity (260), while in mammary tumors, laminin-5 ligation of α6β4 has a strong effect on the activity of NF-κB (184). In addition, it has been reported that osteopontin, an αvβ3 integrin ligand, and fibronectin activate NF-κB (through β3 integrin), while collagen type 1 or β1 integrin ligation are unable to induce NF-κB activation in endothelial cells (254). This is in contrast to monocytes, where β1 integrin ligation is sufficient to activate NF-κB. It is thus most likely that the effects on NF-κB activity and on gene expression induced by the interaction of a given cell with a particular ECM depends on the repertoire of integrins present on this cell, and the composition of the ECM to which it adheres.

The first discovered function of ECM-induced NF-κB activity was regulation of survival. Overexpression of the non-degradable form of IκBα (that blocks NF-κB nuclear translocation and activation) abolishes αvβ3 integrin-mediated endothelial cell survival, indicating that αvβ3 integrin-induced NF-κB activity can play a pro-survival role (254). Furthermore, the same study

reports that although NF-κB activity is required for the pro-survival effects of osteopontin (αvβ3 ligand) and vitronectin on endothelial cells, it is not involved in fibronectin-, laminin-, and collagen I-induced cell survival (254). Thus, only specific ECM induce integrin-mediated survival that is dependent on NF-κB. It has also been reported that NF-κB activation by engagement of α6β4 integrins to autocrine laminin-5 leads to increased survival of mammary tumor cells and to acquisition of anchorage independence (184). Thus, this work suggests that the laminin-5 – α6β4 – NF-κB pathway provides protection against anoikis in mammary epithelial cells (184).

By contrast, it has been reported that NF-κB antisense oligonucleotides do not affect viability but instead inhibit differentiation of endothelial cells cultured on ECM (253). However, as this report does not show the effects of these antisense oligonucleotides on NF-κB protein levels, it is difficult to establish to which extent ECM-induced NF-κB activity is affected by this treatment. Therefore, these latter data remain to be confirmed.

1.4. Pathways involved in the control of NF-κB activity

As indicated above, activation of NF-κB can be regulated at several levels. Two main levels of NF-κB activation have been defined: the first occurs in the cytoplasm, upon degradation of the inhibitory IκB proteins that allows the nuclear translocation of NF-κB, while the second takes place in the nucleus, regulating the transcriptional activity of NF-κB (215; 223; 225). A multitude of signaling pathways generated by a large array of stimuli have been reported to regulate one or both levels controlling NF-κB activity. These pathways include signaling proteins such as protein kinase A (PKA), casein kinase II (CKII), protein kinase C (PKC), PI3K, Akt and ERK (215; 223; 225; 261). We will focus on the signaling pathways that seem to be activated in pancreatic beta cells by 804G-ECM (see Paper 1): the PI3K-Akt signaling module and the MAP kinase ERK pathway.

1.4.1. PI3K-Akt pathway and NF-κB activity

In a study aimed at identifying signaling pathways utilized by the cytokine IL-1β to activate NF-κB, it was observed that IL-1β induces an increase in PI3K activity, as well as a physical interaction between IL-1 receptor and the p85 subunit of PI3K (262). Furthermore, inhibition of PI3K activity alters IL-1β-induced DNA binding and transcriptional activation of NF-κB, as assessed by EMSA and reporter gene assay, indicating that activation of NF-κB by IL-1β requires PI3K. In an another study, it was reported that PI3K inhibitors inhibit IL-1β-induced p65 phosphorylation and NF-κB-dependent gene expression (263). These results suggest that PI3K can be involved in IL-1β-induced p65 transactivation potential (263). A role for Akt in NF-κB activation was reported in 1999 by four different groups (246; 263-265). It was observed that overexpression of Akt (wild-type or constitutive active form) induces NF-κB activation (263; 264). Furthermore, the involvement of Akt in NF-κB activation induced by PDGF or by TNF was also reported (246; 265). Numerous groups have since reported a role for Akt in NF-κB activation induced by diverse stimuli. However, there are also some studies that show that PI3K and/or Akt are not involved in the regulation of NF-κB activity (266; 267). It has been proposed that the role of Akt in NF-κB activity is cell-type dependent, and that the IKKα/IKKβ ratio may be a factor that determines to which extent Akt activity influences NF-κB activity (268). Rosmashkova and colleagues (246) were the first to report an anti-apoptotic role of the PI3K/Akt/NF-κB signaling pathway. Since, several stimuli (including oncogenes, PDGF and TGF-β1) have been reported to induce the anti-apoptotic PI3K/Akt/NF-κB pathway in diverse cell types (159; 246; 269). In the context of the ECM, treatment of cells with PI3K inhibitors (LY294002 and/or wortmannin) has been reported to prevent nuclear translocation of p65 upon adhesion to fibronectin (260) and to inhibit the transcriptional activity of NF-κB induced by an activating β1 integrin antibody (175). However, it has been shown that osteopontin-induced NF-

κB activity is not altered upon treatment with LY294002 (254), suggesting that the involvement of PI3K in integrin-induced NF-κB activity is not a general mechanism. Studies investigating a possible role of Akt in ECM and/or integrin-dependent NF-κB transcriptional activity have not yet been reported.

In summary, both PI3K and Akt have been reported to be involved in IL-1β-induced NF-κB activity, while only PI3K has been reported to be involved in fibronectin and/or β1 integrin-induced NF-κB activity to date. In addition, the ability of PI3K and/or of Akt to mediate the activation of NF-κB induced by diverse stimuli appears to be cell-type specific. Finally, the PI3K/Akt/NF-κB pathway has been reported to play an anti-apoptotic role in different contexts.

1.4.2. ERK pathway and NF-κB activity

ERK has been shown to be involved in NF-κB activation induced by different stimuli, including TNFα (270), LPS (271), IL-1β (272) and TGF1-β (269). ERK has also been involved in serum-induced, calcium-mediated NF-κB activation (273). Interestingly, it has been proposed that ERK is involved in the expression of some, but not all IL-1β-induced, NF-κB-dependent genes (274). In particular, this report proposes that ERK is required for the induction of the "late" NF-κB-dependent genes, and therefore that ERK is an important temporal regulator of NF-κB-dependent gene regulation (274). ERK-induced NF-κB activity has been reported to play an anti-apoptotic role. Interestingly, the anti-apoptotic effect of ERK-induced NF-κB activity appears to be highly dependent on the nature of the pro-apoptotic stimuli (181). In the context of ECM-induced NF-κB activity, ERK has been shown to be partially involved in α4β1-induced NF-κB binding to DNA in THP-1 cells (211). However, several groups have reported that ECM and/or integrin-induced NF-κB activity do not depend on MEK1 activity (175; 254; 260). Thus, the role of the MAP kinase ERK pathway in integrin-dependent NF-κB activity may be cell type-dependent and/or context-dependent.

In summary, ECM has been shown to be able to induce expression of genes through induction of the transcriptional activity of NF-κB. The signaling molecules FAK, PI3K, Akt and ERK have all been reported to be able to activate NF-κB. Several signaling molecules have been shown to be involved in ECM-induced NF-κB activity, and these include PI3K and ERK.

1.5. NF-κB and the pancreatic beta cell

As described above, type 1 diabetes mellitus is an auto-immune disease causing specific destruction of pancreatic beta cells. Pro-inflammatory cytokines, in particular IL-1β, IFN-γ and TNF-α, that are released by macrophages, monocytes and natural killer cells, play important roles in beta cell dysfunction and destruction associated with type 1 diabetes. Activation of NF-κB by pro-inflammatory cytokines IL-1β and TNFα in the pancreatic beta cell is well documented, and this pathway is now considered as one of the major factors involved in beta cell dysfunction and death in type 1 diabetes (29; 32).

Increased production of nitric oxide (NO), due to induction of inducible nitric oxide synthase (iNOS), is thought to be an important signal in cytokine-induced cell death (29). IL-1β-induced iNOS expression is inhibited by treatment of cells with proteasome inhibitors that block NF-κB activation (diethyldithiocarbamate DETC, and PDTC) (275), suggesting that NF-κB plays a critical role in IL-1β-induced iNOS expression and in cytokine-induced beta cell death. Adenoviral transfer of non-phosphorylatable and non-degradable IκBα variant (hence called Ad-IκB), that sequesters NF-κB in the cytoplasm, has also been used as a more specific means than proteasome inhibitors to assess the role of NF-κB in beta cells. Ad-IκB prevents IL-1β-induced dysfunction, NO production and Fas-triggered, IL-1β-mediated induction of apoptosis in human islets, indicating that NF-κB is involved in the deleterious effects of IL-1β leading to beta cell dysfunction and death (276). The protective effect of Ad-IκB against cytokine (IFNγ + IL-1β)-induced apoptosis has also been reported for purified rat beta cells (277). MIN6 clones that stably express the same IκB variant are protected against cytokine (IL-1β + IFNγ + TNFα)-induced apoptosis compared to control clones (278); and peptide-mediated transduction of the IKK inhibitor Nemo-binding domain (NBD), that inhibits IL-1β-induced NF-κB activation, blocks IL-1β-mediated dysfunction of mouse islets (279). Furthermore, *in vivo* transduction of this peptide in mice prior to isolation prevents loss of function and viability of islets due to isolation (279). Thus, these papers indicate that cytokine-induced NF-κB activity plays a pro-apoptotic role in pancreatic beta cells. However, there is one report that indicates that cytokine (TNFα)-induced NF-κB activity can have anti-apoptotic effects on the mouse insulinoma cell line MIN-6 and in primary mouse islets (280). Indeed, TNFα alone is not able to induce apoptosis of beta cells. However, treatment of cells with the proteasome inhibitor MG132 (that inhibits NF-κB) or with Ad-IκB sensitizes cells to TNFα-induced apoptosis, suggesting that TNFα-induced activation of NF-κB protects beta cells (MIN-6, mouse islets) from TNFα-mediated apoptosis (280).

To better understand the mechanisms of this cytokine-NF-κB connection in cytokine-induced beta cell dysfunction and death, microarray analysis has been performed on purified primary rat beta cells that were treated or not with cytokines (IL-1β + IFNγ), and that were

infected with either a control adenovirus or with Ad-IκB (281). This approach led to the identification of 66 cytokine-modified and NF-κB-regulated genes in beta cells. Most notably, it was found that cytokine-induced NF-κB activation leads to an increase in the expression of pro-apoptotic genes (such as c-Myc, Fas and iNOS) and of chemokines and cytokines, that might contribute to the activation and the recruitment of inflammatory cells to the area of insulitis. Furthermore, it was observed that cytokine-induced NF-κB activity leads to a decrease in the expression of genes involved in the maintenance of the differentiated beta cell functions, such as Pdx-1, Isl-1 and Glut-2. Finally, cytokine-induced modifications of the expression of several transcription factors, such as C/EPBβ, C/EBPδ, c-Myc and Isl-1 were found to be NF-κB-dependent (281). In summary, these data suggest that NF-κB is a key switch regulator of transcription factors and gene networks controlling cytokine-induced beta cell dysfunction and death occurring in the pathogenesis of type 1 diabetes.

Until recently, IL-1β production and release by islets was considered to be limited to type 1 diabetes. However, in a recent breakthrough report, it was suggested that this mechanism is also involved in the development of type 2 diabetes (282). As described above (Part I, § 1.2.2.), glucotoxicity is considered as one of the main mechanisms leading to apoptosis in type 2 diabetes. Maedler and colleagues have reported that culture of human islets with high glucose concentrations induces secretion of IL-1β by the beta cells themselves, leading to their dysfunction as well as an increase in beta cell death (282). Inhibition of IL-1β signaling (using IL-1Ra) blocks high glucose- and IL-1β-induced NF-κB activity; and blockade of IL-1β signaling and of NF-κB activity (with the proteasome inhibitor PDTC) block the deleterious effects of high glucose concentrations on both beta cell function and survival. Thus, these results indicate that release of IL-1β by beta cells cultured in high glucose concentrations is followed by NF-κB activation and, ultimately, by impaired beta cell function and survival (282). In addition, IL-1β-producing cells are observed in pancreatic sections of poorly controlled type 2 diabetic patients but not in non-diabetic control subjects (282). The diabetes-prone gerbil *Psammomys obesus* is an animal that develops type 2 diabetes upon high-energy diet (21). Interestingly, IL-1β expression is induced in the beta cells of the diabetes-prone *Psammomys obesus* fed with a high-energy diet, but not in the diabetes-resistant counterparts (282). These observations indicate that the effect of high glucose on IL-1β production leading to NF-κB activation in the beta cell might be a physiologically relevant mechanism involved in the development of type 2 diabetes. Furthermore, it was recently reported that two drugs that inhibit NF-κB activity (pioglitazone and sodium salicylate) protect human beta cells against impaired function and apoptosis induced by IL-1β and high glucose (283).

As described above, NF-κB is known to play both pro- and anti-apoptotic roles in diverse cell types. In particular, it seems that the context, nature, duration and dose of the stimuli leading to activation of NF-κB determine the biological outcome of NF-κB-dependent

gene expression (230; 284-286). This seems to also be the case for the pancreatic beta cell. Indeed, in the studies described above, beta cells were exposed for prolonged times to high doses of cytokines. However, it has been reported that when beta cells are exposed for a limited period (24h) to low concentrations of IL-1β, the protective effects of NF-κB-regulated genes preponderate and beta cells are protected against conditions that cause necrosis (287). This suggests that the effects of IL-1β-induced NF-κB activity are dose- and/or duration-dependent. This concept is supported by a recent report indicating that the first phase of IL-1β-induced NF-κB activity leads to an increase of beneficial beta cell defense/repair protein expression (288). By contrast, the second phase of NF-κB activity induced by IL-1β seems to be harmful because it then leads to a sustained decrease of specific beta cell proteins like insulin, Glut-2 and Pdx-1 with a concomitant increase of other "aspecific" proteins and iNOS transcription (288). However, in this study, the effects of IL-1β-induced NF-κB activity were assessed using the protease inhibitor TPCK, and thus remain to be confirmed with more specific means to inhibit NF-κB.

Other stimuli than cytokines have been shown to activate NF-κB in pancreatic beta cells, such as depolarization and Ca^{2+} influx (289). However, the consequences of this NF-κB activity remain to be characterized. GLP-1 has been reported to induce the NF-κB DNA-binding activity and the expression of the anti-apoptotic NF-κB target genes IAP-1 and Bcl-2 in INS-1 cells (190). Interestingly, GLP-1-induced protection of beta cells from glucolipotoxicity is abolished upon treatment of cells with the NF-κB inhibitor Bay 11-7082, suggesting that NF-κB mediates the anti-apoptotic effect of GLP-1 on beta cells cultured in glucolipotoxic conditions (190). Stable overexpression of c-FLIP in the mouse beta cell line βTc-Tet leads to protection of mouse beta cells against cytokine-induced caspase-8 activation and apoptosis, as well as to an enhanced NF-κB activity (290). Although this report does not show evidence that these two phenomena are related, it is proposed by the authors that protection of beta cells against cytokine-induced apoptosis by c-FLIP overexpression may be linked to its effect on NF-κB activity (290). Finally, in a very recent report Norlin and colleagues report the generation of mice harbouring beta-cell specific overexpression of a non-phosphorylatable mutant of IκBα that inhibits NF-κB activity (291). Interestingly, glucose-stimulated insulin secretion was impaired in the transgenic mice, while beta cell survival was not affected (291).

In summary, the majority of studies assessing the effects of IL-1β-induced NF-κB activity report a deleterious effect of this activity for pancreatic beta cell function and survival. Furthermore, this signaling module has been suggested to be potentially involved in the pathogenesis of both types of diabetes. Little is known about the functional consequences of NF-κB activity induced by stimuli other than cytokines. However, these data suggest the functional consequences of NF-κB activity induced by stimuli other than cytokines may differ considerably from the deleterious effects of cytokine-induced NF-κB activity on the beta cell.

Finally, no studies on the possible effects of ECM and/or integrins on NF-κB activity have been reported yet for the pancreatic beta cell.

2. Specific Aims of Paper 2

Gene expression analysis performed with gene arrays (as shown in Paper 1) and additional experiments performed in our laboratory with Affymetrix microarrays, followed by real-time RT-PCR validation have shown that 804G-ECM induces overexpression of a number of genes, including IκBα, NF-κB1 (p105), and the chemokines Mob-1 (also called IP-10) and GRO-1 (growth-related oncogene 1). As these genes have all been reported to be induced by NF-κB, we hypothesized that 804G-ECM might induce the transcriptional activity of NF-κB. Furthermore, we hypothesized that activated NF-κB might mediate at least some of the effects of 804G-ECM on the pancreatic beta cell. The possibility that NF-κB might mediate some of the *positive* effects of 804G-ECM was most intriguing, as this transcription factor has mostly been reported to play deleterious roles in the pancreatic beta cell (in the context of cytokine-induced NF-κB activity). However, there are some evidences that indicate that NF-κB activity induced by stimuli other than cytokines can lead to biological outcomes that differ from those induced by cytokine-induced NF-κB activity.

While this work was in progress, our lab started to compare the organization of the actin cytoskeleton in cells cultured on 804G-ECM with cells cultured on control substrate (pLL). It was found that 804G-ECM induces a remodeling of the actin cytoskeleton. 804G-ECM can thus affect the following biological functions in the pancreatic beta cell: spreading, actin cytoskeleton remodeling, glucose-stimulated insulin secretion and cell survival. Therefore, the specific aims of this second part of the thesis were to:

- establish whether 804G-ECM is able to induce nuclear translocation and/or transcriptional activity of NF-κB;

- investigate whether 804G-ECM-induced NF-κB activity plays a role in the following effects of 804G-ECM on the pancreatic beta cell: cell spreading, actin cytoskeleton remodeling, glucose-stimulated insulin secretion and cell survival

- determine whether the MAP kinase ERK pathway and the PI3K-Akt signaling module are involved in 804G-ECM-induced NF-κB activity.

3. Paper 2

“ Activation of NF-κB by Extracellular Matrix Is Involved in Spreading and Glucose-stimulated Insulin Secretion of Pancreatic Beta Cells”

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Activation of NF-κB by Extracellular Matrix Is Involved in Spreading and Glucose-stimulated Insulin Secretion of Pancreatic Beta Cells*

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Laminin-5-rich extracellular matrix derived from 804G cells (804G-ECM) engages β1 integrins to induce spreading, improve glucose-stimulated insulin secretion (GSIS), and increase survival of pancreatic beta cells. The present study examines whether 804G-ECM activates the transcriptional activity of NF-κB and the involvement of NF-κB in those effects of 804G-ECM on pancreatic beta cells. 804G-ECM induces nuclear translocation and the DNA binding activity of the p65 subunit of NF-κB. 804G-ECM-induced nuclear translocation of NF-κB was weak as compared with that induced by interleukin-1β. Transient 804G-ECM-induced DNA binding activity of NF-κB (peak at 2 h) and overexpression of NF-κB target genes IκBα and NF-κB1(p105) (peak at 4 h) were observed. When NF-κB was inhibited by an inhibitor of IκBα phosphorylation (Bay 11-7082) or by a recombinant adenovirus expressing the nonphosphorylatable form of IκBα, 804G-ECM-induced cell spreading and actin cytoskeleton organization were reduced. GSIS from cells on 804G-ECM was inhibited 5-fold, whereas cell survival was not affected. In summary, the results indicate that 804G-ECM induces a transient and moderate NF-κB activity. This study shows for the first time that ECM-induced NF-κB activity is necessary in maintaining GSIS, although it does not affect survival of pancreatic beta cells. The effects of ECM-induced NF-κB activity contrast with the deleterious effects of cytokine-induced NF-κB activity. It is proposed that transient and moderate NF-κB activity is essential for proper function of the pancreatic beta cell.

Engagement of cells by cognate components of the extracellular matrix (ECM)[1] is crucial for various biological processes, including cell adhesion, spreading, proliferation, differentiation, migration, apoptosis, and gene induction (1). This engagement involves cell adhesion mediated by integrins, a family of heterodimeric molecules composed of an α and a β subunit, with a long extracellular domain binding to the ECM, and a short cytoplasmic domain associating with the actin cytoskeleton and affiliated proteins (2). ECM is an important component of the pancreatic islet microenvironment. It is a dynamic complex of different molecules that serve as a cellular scaffold regulating both differentiation and survival.

Our group has reported recently (3) that the laminin-5-rich ECM secreted by 804G cells (804G-ECM) induced increased spreading and improved insulin secretion in response to glucose in purified pancreatic beta cells. It was subsequently shown that this ECM has a beneficial effect on cell survival and that it activates intracellular signaling pathways involving the signaling proteins focal adhesion kinase, Akt/PKB, and ERK (4). It has been shown that its effects on pancreatic beta cells (spreading, glucose-stimulated insulin secretion, and survival) are mediated by the engagement of β1 integrins to laminin-5 (3, 4).[2] Furthermore, it was found that plating pancreatic beta cells on 804G-ECM causes overexpression of the inhibitor of NF-κB (IκBα) (4), which is a well established NF-κB target gene. This finding led us to hypothesize that 804G-ECM might activate the NF-κB signaling pathway, leading to the expression of a subset of genes, which might be involved in the effects of 804G-ECM on the pancreatic beta cell. Engagement of integrins to different ECM molecules (vitronectin, fibronectin, and laminin) has been shown to activate NF-κB (5–9) and to promote an NF-κB-dependent program of gene expression (10, 11). However, this has not yet been reported for the pancreatic beta cell nor indeed for primary, nondividing cells.

NF-κB is a transcription factor that plays a pivotal role in many cellular responses to environmental changes, including control of the host immune and inflammatory response, apoptosis, cell cycle progression, cell migration, development, induction of proliferation, and differentiation (12, 13). In nonstimulated cells, NF-κB remains in the cytoplasm, where it is bound to and inactivated by inhibitory IκB subunits (14). Upon stimulation of the cell (with pro-inflammatory cytokines for example), signaling pathways lead to phosphorylation of the IκB proteins that are subsequently recognized by the ubiquitin ligase machinery and are targeted for degradation. The freed NF-κB heterodimers translocate to the nucleus, where they bind to specific sequences in the promoter or enhancer regions of target genes (reviewed in Ref. 12). Activated NF-κB can then

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[1] The abbreviations used are: ECM, extracellular matrix; GSIS, glucose-stimulated insulin secretion; NF-κB, nuclear factor-κB; IκBα, IκBβ, inhibitor of NF-κB; IκBαnp, nonphosphorylatable IκBa mutant; ERK, extracellular signal-regulated kinase; PI3K, phosphatidylinositol 3-kinase; pLL, poly-L-lysine; PKB, protein kinase B; ELISA, enzyme-linked immunosorbent assay; MAP, mitogen-activated protein; PBS, phosphate-buffered saline; GFP, green fluorescent protein; TUNEL, terminal dUTP nick-end labeling; IL, interleukin.

[2] G. Parnaud, unpublished results.

be down-regulated by multiple mechanisms, including the well characterized feedback pathway involving newly synthesized IκBα proteins (12, 15, 16).

In the pancreatic beta cell, NF-κB is considered as an important transcription factor mediating IL-1β-induced signal transduction and regulating groups of genes contributing to death and dysfunction (17). Indeed, inhibition of NF-κB has been shown to protect against the deleterious effects of IL-1β in purified mouse islets (18), rat beta cells (19), and human islets (20, 21). Given that NF-κB has thus been considered previously to be pro-apoptotic in the beta cell, the possibility that activation of NF-κB signaling by 804G-ECM may be beneficial for beta cell function and/or survival was quite intriguing. The aims of this study were to investigate whether 804G-ECM activates the NF-κB transcriptional activity in primary pancreatic beta cells, to study the kinetics of this activity, and to determine the involvement of this pathway on the effects of 804G-ECM on the pancreatic beta cell. Here we show that 804G-ECM induces transient nuclear translocation of NF-κB and its transcriptional activity in pancreatic beta cells, which is followed by overexpression of IκBα and NF-κB mRNAs. Blocking the MAP kinase ERK pathway with PD98059 inhibited the DNA binding activity of NF-κB and overexpression of IκBα, whereas the PI3K inhibitor LY294002 did not. We report, for the first time, that long term blockage of NF-κB activity disrupts cytoskeleton remodeling, inhibits spreading induced by 804G-ECM, and reduces glucose-stimulated insulin secretion. Furthermore, we show that induction of NF-κB activity by 804G-ECM is not involved in its control of pancreatic beta cell survival.

MATERIALS AND METHODS

Reagents and Antibodies—Bay 11-7082 (an inhibitor of IκBα phosphorylation) was purchased from BioMol Research Laboratories (Hamburg, Germany). PD98059 (a specific MEK1 inhibitor) and LY294002 (a phosphatidylinositol 3-kinase (PI3K) inhibitor) were purchased from Calbiochem, and both were used at final concentration of 50 μM as described previously (4). The polyclonal antibodies against the p65 subunit of NF-κB (C-20) and against the inhibitors of NF-κB (IκBα, C-21; IκBβ, C-20) were from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal anti-actin was purchased from Chemicon International (Temecula, CA). Anti-mouse horseradish peroxidase and anti-rabbit horseradish peroxidase antibodies were from Amersham Biosciences. Alexa Fluor® 546 phalloidin was purchased from Molecular Probes.

Islet Isolation and Beta Cell Purification—All experiments were performed on primary pancreatic beta cells sorted from adult rat islet cells by autofluorescence-activated flow cytometry. Islets of Langerhans were isolated by collagenase digestion of pancreas from male Wistar rats (weighing 150–200 g), followed by Ficoll purification using a modification of procedures described previously (22). Cell preparation was performed as described previously (3). Pancreatic beta cells were then separated from non-beta cells by autofluorescence-activated sorting using a fluorescence-activated cell sorter, FACStar-Plus (BD Biosciences), as described previously (22). This purification procedure yields a population consisting of >95% beta cells (3).

804G-ECM Matrix Preparation—The 804G cells were the kind gift of Desmos (San Diego, CA). They were grown in Dulbecco's modified Eagle's medium containing 10% fetal calf serum and 5.6 mM glucose. At confluence, cells were rinsed and maintained for a further 3 days in the same medium in the absence of fetal calf serum. Conditioned medium (referred to hereafter as 804G-ECM) was collected, centrifuged at 120 × *g* for 10 min to remove any detached cells and debris, filtered through a 0.22-μm Millipore filter, and frozen at −20 °C for later use.

Coating of Plastic Dishes with pLL and 804G-ECM—Aliquots (60 μl) of pLL (0.1 mg/ml) or of 804G-ECM were layered at the center of 35-mm culture Petri dishes (adherent dishes for mammalian cell culture). Dishes were kept in a damp box at 37 °C for 18–20 h before being rinsed three times with sterile H_2O and air-dried. Dishes coated with pLL were used as controls.

Cell Culture—Sorted beta cells were washed twice in 10–15 ml of sterile Dulbecco's modified Eagle's medium (Invitrogen) containing 10% fetal calf serum, 11.2 mM glucose, 110 μg/ml sodium pyruvate and supplemented with 110 units/ml penicillin, 110 μg/ml streptomycin, and 50 μg/ml gentamycin, followed by centrifugation for 10 min at 130 × *g*. Aliquots of 3×10^5 cells were seeded in nonadherent 100-mm diameter Petri dishes containing 9 ml of medium. Cells were then incubated for 20 h at 37 °C to allow full recovery of any cell surface molecules that may have been lost or damaged during islet isolation or cell purification. After recovery, cells were resuspended at a density of 4×10^5 cells/ml, and aliquots of 50 μl were plated as droplets on plastic dishes coated with pLL or with 804G-ECM.

NF-κB Activation—The cellular localization of NF-κB in various conditions was analyzed by immunofluorescence. Cells were fixed with 4% paraformaldehyde (20 min, room temperature) and permeabilized with 0.5% Triton X-100 (5 min, room temperature), and immunofluorescence for NF-κB was performed. The percentage of cells with dominant nuclear NF-κB staining was determined (five different fields were examined for each condition). DNA binding activity of NF-κB was quantified using an ELISA-based kit (Trans-AM NF-κB p65 from Active Motif, Rixensart, Belgium) using attached oligonucleotides corresponding to an NF-κB consensus site and detected by an anti-p65 subunit antibody according to the manufacturer's instructions.

Western Blot Analysis—To analyze IκBα and IκBβ protein expression, attached cells were washed with ice-cold PBS supplemented with 1 mM sodium vanadate and protease inhibitors and lysed in sample buffer 1× (62 mM Tris-Cl, pH 6.8, 2% SDS, 5% glycerol, 1% 2-mercaptoethanol). Protein concentrations were determined with the Amido Black method (23), and equal amounts of total protein were loaded for SDS-PAGE. All samples, after separation on an SDS-polyacrylamide gel, were electroblotted onto nitrocellulose membranes (Schleicher & Schuell) for immunoblotting with the appropriate antibody. An ECL protein detection kit (Amersham Biosciences) and a Kodak image station were used for visualization of the bands.

Quantitative Real Time PCR—RNA was isolated using RNeasy mini kit (Qiagen). cDNA was synthesized with Superscript II (Invitrogen), using 1 μg of total RNA in a 20-μl reaction volume. The double-stranded DNA-specific dye SYBR Green I (Eurogentech, Belgium) and fluorescein (Bio-Rad) were incorporated into the PCR buffer (qPCR core kit, Eurogentech) to allow for quantitative detection of the PCR product. The results were analyzed using the iCycler iQ System (Bio-Rad). The housekeeping gene L3 was used as an internal control. The primers used were as follows: NF-κB forward 5′ GTC TAG CAA TCA CGG CTG CA 3′, NF-κB reverse 5′ CTC AAG CCA CCA TAC CCC AA 3′; IκBα forward 5′ TGC TGA GGC ACT TCT GAA AGC 3′, IκBα reverse 5′ TCC TCG AAA GTC TCG GAG GTC 3′.

Inhibition of NF-κB Activity—Two approaches were used to inhibit NF-κB activity as follows: use of recombinant adenoviruses expressing GFP (control virus) or mutated nondegradable IκBα (IκBαnp), and pharmacological inhibition of IκBα phosphorylation with Bay 11-7082 (24). The recombinant adenoviruses were a kind gift from Dr. Wrede, University of Regensburg, Germany (19, 25). The adenoviruses were titrated using Adeno-X™ Rapid Titer kit (Clontech) according to the manufacturer's instructions. Cells (10^5 cells/ml) were infected in suspension for 2 h at 37 °C with 2300 infectious units/ml, washed twice with culture medium, and left in suspension for 24 h before plating them on pLL or on 804G-ECM-coated dishes. To investigate the effect of the inhibition of NF-κB activity using Bay 11-7082, cells were pretreated for 1 h with 2.5 and 5 μM Bay 11-7082 before plating them on pLL- or on 804G-ECM-coated dishes. Me_2SO was added to the controls at the same final concentration as used for the inhibitors. Cells were then cultured in the continued presence of the inhibitor. The analyses of the effects of IκBαnp-expressing virus and of Bay 11-7082 on NF-κB nuclear translocation, cell spreading, cell function (insulin secretion), and cellular death were performed in parallel.

Measurement of Spreading of Cells—After 24 h of culture and treatment of cells as described above, cells were fixed with 4% paraformaldehyde (20 min, room temperature) and stained with Evans Blue. Phase-contrast views of different fields were photographed, and spreading of cells was quantified using ScionImage™ software (Frederick, MD).

F-actin Cytoskeleton Visualization—After 24 h of culture and treatment of cells as described above, cells were fixed with 4% paraformaldehyde (20 min, room temperature) and permeabilized with 0.5% Triton X-100 (4 min at room temperature). After blocking with PBS + 0.5% bovine serum albumin, cells were incubated for 30 min with Alexa Fluor® 546 phalloidin (5 units/ml), subsequently rinsed, and mounted under glass coverslips. The preparations were observed with a confocal microscope.

Analysis of Cell Death by TUNEL—Attached cells were washed with PBS and fixed with 4% paraformaldehyde (20 min, room temperature). After permeabilization with 0.5% Triton X-100 (5 min, room temperature), the TUNEL assay (with fluorescein-dUTP detecting the free

3-OH strand breaks resulting from DNA degradation) was performed with the In Situ Cell Death Detection kit, according to the manufacturer's instructions (Roche Applied Science). The preparations were then rinsed with PBS and incubated (15 min, room temperature) with 1 μg/ml Hoechst 33342 to allow detection of nuclei and to facilitate the analysis. The quantification of dead cells was performed by using an Axiocam fluorescence microscope.

Insulin Secretion Assay and Insulin Content—Cells were washed three times with a modified Krebs-Ringer bicarbonate HEPES buffer (KRBH: 125 mM NaCl, 4.74 mM KCl, 1 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 5 mM $NaHCO_3$, 25 mM HEPES, pH 7.4, 0.1% bovine serum albumin) supplemented with 2.8 mM glucose and preincubated with this same buffer for 1 h at 37 °C. Cells were then incubated for 1 h at 37 °C with KRBH containing 2.8 mM glucose (basal secretion), followed by 1 h at 37 °C with KRBH containing 16.7 mM glucose (stimulated secretion). The incubation buffer was recovered, and the cells were extracted with acid/ethanol. The buffer was centrifuged to remove any detached cells and debris. Aliquots were stored at −20 °C for subsequent insulin measurement performed by radioimmunoassay using the charcoal separation technique described previously (26). GSIS is expressed as secreted insulin upon glucose challenge (16.7 mM glucose, for 1 h), as a percentage of insulin content. Insulin content is expressed as the quantity of insulin extracted at the end of the stimulation test. The total protein levels after 24 h of culture in all conditions used were quantified using the Amido Black method (23), as described above.

Presentation of Data and Statistical Analysis—Unless stated otherwise, data are presented as mean ± S.E. for "*n*" independent experiments, and levels of significance for differences between groups were assessed by Student's *t* test for unpaired groups.

RESULTS

804G-ECM Induces NF-κB Nuclear Translocation—This study was performed with laminin-5-rich extracellular matrix derived from 804G rat bladder carcinoma cells (804G-ECM). 804G-ECM induces attachment and spreading of rat primary beta cells, because of engagement of β1 integrins by laminin-5 contained in this ECM (3, 4). 804G-ECM is thus used as a validated model to study signaling pathways activated by this engagement.

In the canonical NF-κB pathway, one of the first steps involved in NF-κB-dependent gene regulation is the phosphorylation and subsequent degradation of the inhibitor of NF-κB (IκBα)), leading to the nuclear translocation of the p65 subunit of NF-κB (RelA). To investigate a possible involvement of 804G-ECM in NF-κB transcriptional activity, the cellular localization of NF-κB after short term exposure (1 h) to 804G-ECM compared with control (pLL-coated dishes) was assessed by immunofluorescence for the p65 subunit of NF-κB (Fig. 1*A*). The number of cells with nuclear NF-κB localization was quantified (Fig. 1*B*). 804G-ECM induced a significant increase in the number of cells with nuclear NF-κB (19%) compared with control (less than 5%, Fig. 1*B*). The cytoplasmic staining for NF-κB was still apparent in all cells on 804G-ECM, suggesting that only a fraction of the total pool of cytoplasmic NF-κB is translocated into the nucleus in response to 804G-ECM (Fig. 1*A*). By contrast, treatment of cells for 20 min with 2 ng/ml IL-1β induced the nuclear translocation of p65 in all treated cells, and this was complete as there was no more cytoplasmic staining for NF-κB (not shown).

804G-ECM Induces NF-κB Transcriptional Activity—To assess whether DNA binding activity of NF-κB is induced by 804G-ECM, the amount of p65 complexes binding to oligonucleotides containing an NF-κB consensus binding site was quantified by ELISA. The DNA binding activity of NF-κB in cells cultured on 804G-ECM-coated dishes as compared with control was increased after 1 h of culture, and this increase was significant after 2 h of culture (Fig. 2). This activity was decreased after 4 h of culture suggesting that 804G-ECM-induced NF-κB DNA binding activity is transient.

It is well established that activated NF-κB induces expression of IκBα. Newly synthesized IκBα binds to NF-κB, providing a negative feedback loop for the NF-κB signaling pathway (12). Activated NF-κB is also able to induce the expression of NF-κB1 (p105) in several systems (11, 27). To investigate whether 804G-ECM is able to induce NF-κB transcriptional activity, IκBα and NF-κB1 mRNA levels were quantified by real time PCR in cells cultured on pLL- or on 804G-ECM-coated dishes. IκBα mRNA levels were increased after 2 h on 804G-ECM, and both IκBα and NF-κB1 mRNA levels were significantly increased by 804G-ECM after 4 and 8 h of culture to return to control (pLL) levels at 24 h (Fig. 3, *A* and *B*). The kinetics of 804G-ECM-induced IκBα and NF-κB1 gene expression thus correlate with those of NF-κB DNA binding activity (Fig. 2). In summary, these data indicate that 804G-ECM induces NF-κB transcriptional activity transiently, most probably as a consequence of feedback inhibition resulting from increased expression of IκBα.

Regulation of IκBα and IκBβ Protein Levels by 804G-ECM—To investigate whether 804G-ECM induces IκBα and/or IκBβ degradation, Western blots were performed with protein extracts from cells cultured for 1 h on pLL or on 804G-ECM-coated dishes. Quantification of the resulting bands normalized to actin showed that there was no significant decrease of IκBα and IκBβ protein levels on 804G-ECM as compared with pLL after 1 h of culture (Fig. 4).

IκBα and IκBβ protein levels were also analyzed after 24 h of culture on pLL- or on 804G-ECM-coated dishes. Levels of IκBα

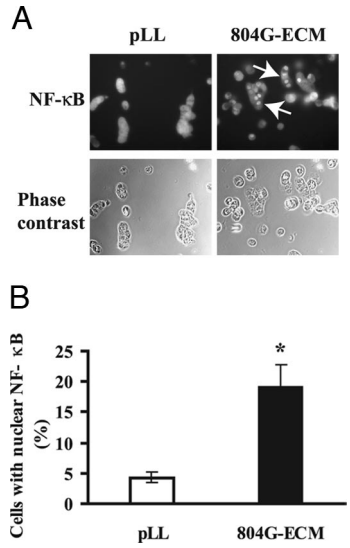

FIG. 1. **804G-ECM induces NF-κB nuclear translocation.** *A*, cells were fixed after 1 h of exposure to pLL (control) or 804G-ECM, and NF-κB was detected by immunofluorescence. *Arrows* show examples of cells with nuclear localization of NF-κB. *B*, the number of cells with nuclear NF-κB localization was quantified as a percent of total. *, $p < 0.05$ relative to pLL ($n = 4$), with a minimum of 100 beta cells examined for each condition in each experiment.

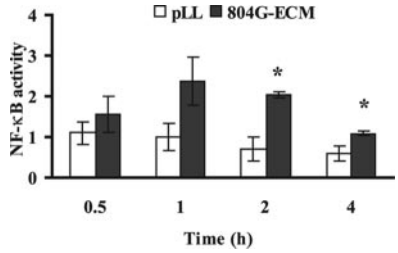

FIG. 2. **Kinetics of NF-κB DNA binding activity induced by 804G-ECM.** NF-κB DNA binding activity (normalized to pLL, 1 h) was measured by ELISA (absorbance, 450–650 nm) after different times of culture on pLL or on 804G-ECM-coated dishes. *, $p < 0.05$ relative to pLL at respective time point ($n = 3$).

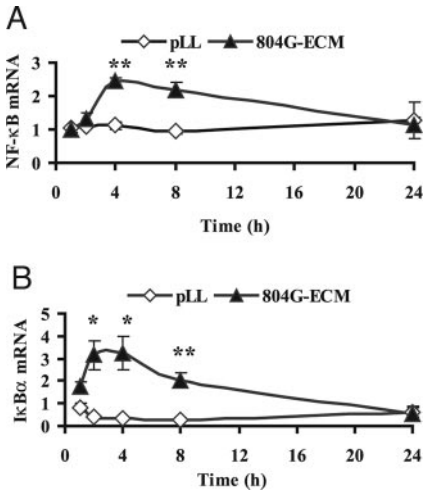

FIG. 3. **NF-κB and IκBα mRNA levels are regulated by 804G-ECM.** Cells were cultured on pLL or on 804G-ECM-coated dishes; mRNA was extracted at the indicated time points, and real time reverse transcription-PCR was performed. The results shown are NF-κB (*A*) and IκBα (*B*) mRNA levels relative to L3 (internal control), normalized to control conditions (cells on respective substrate for 30 min). *, $p < 0.05$; **, $p < 0.005$ relative to pLL at the respective time point ($n = 3$).

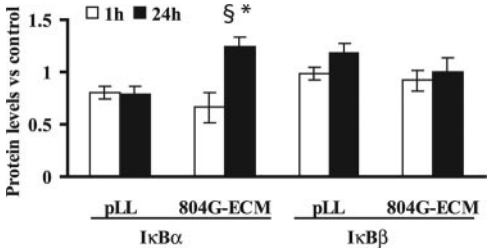

FIG. 4. **Regulation of IκBα and IκBβ protein levels by 804G-ECM.** Cells were cultured for 1 or 24 h on pLL or 804G-ECM-coated dishes; proteins were extracted, and Western blot was performed for IκBα and IκBβ. The resulting bands were scanned, and the density of each band (measured using ScionImage™ software) was normalized to the corresponding actin band. Control, suspended cells. §, $p < 0.05$ *versus* 804G 1 h; *, $p < 0.05$ *versus* pLL 24 h ($n = 3$).

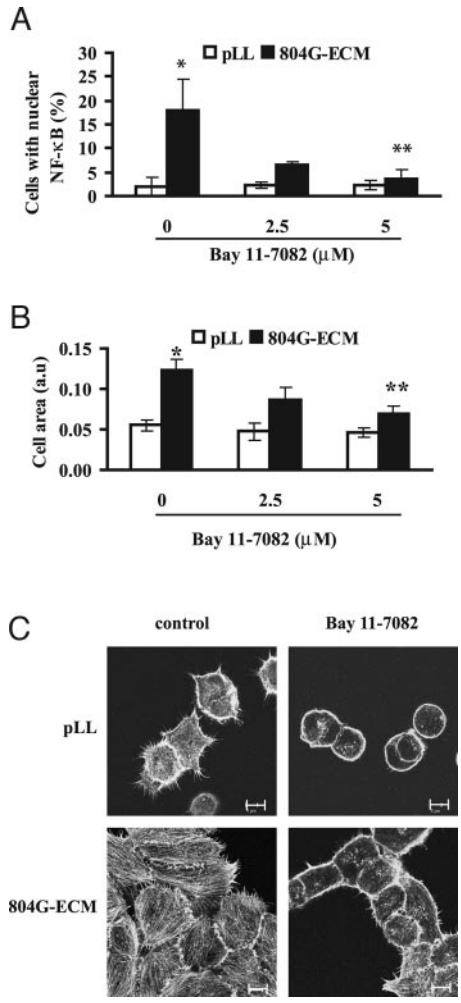

FIG. 5. **Inhibition of NF-κB activity with Bay 11-7082 alters spreading and actin cytoskeleton remodeling.** Cells were preincubated for 1 h with Bay 11-7082 (2.5 or 5 μM), before plating them on pLL or 804G-ECM-coated dishes in the continued presence of the inhibitor and for the indicated times. *A*, effect of Bay 11-7082 on NF-κB nuclear translocation. Cells were fixed after 1 h, and NF-κB was detected by immunofluorescence. The number of cells with a predominant nuclear NF-κB localization was quantified as a percent of total. Results are shown as means ± S.D. ($n = 3$ for control and 5 μM Bay; $n = 2$ for 2.5 μM Bay). *, $p < 0.02$ relative to pLL control; **, $p < 0.02$ relative to 804G control, with a minimum of 100 beta cells examined for each condition in each experiment. *B*, effect of Bay 11-7082 on spreading of cells induced by 804G-ECM. Cells were fixed after 24 h; photographs were taken, and the area of cells was quantified using ScionImage™ software. Cell area is expressed as arbitrary units (*a.u.*). *, $p < 0.005$ *versus* pLL control; **, $p < 0.02$ *versus* 804G control ($n = 3$–4). *C*, effect of Bay 11-7082 (5 μM) on actin cytoskeleton organization. Cells were fixed after 24 h, and F-actin filaments were labeled with Alexa Fluor® 546 phalloidin. *Scale bar* = 10 μm. The confocal plane was set at the basal face (contact with culture surface) of the cells.

protein were significantly increased on 804G-ECM after 24 h of culture as compared with pLL and as compared with 804G-ECM after 1 h of culture (Fig. 4). By contrast, IκBβ protein levels were not altered with time or by the substrate used. Although NF-κB induces expression of IκBα, it does not control expression of IκBβ (28). Therefore, these results suggest that the 804G-ECM-induced IκBα overexpression occurs specifically through NF-κB.

Inhibition of NF-κB Activity; Effects on Spreading and Actin Cytoskeleton Remodeling—To investigate the function of the 804G-ECM-induced NF-κB activity, Bay 11-7082, an inhibitor of IκBα phosphorylation and thus of NF-κB nuclear translocation (24), was used. Bay 11-7082 induced a dose-dependent decrease of 804G-ECM-induced NF-κB nuclear translocation, and a final concentration of 5 μM of Bay 11-7082 was necessary to induce a complete and significant decrease compared with control condition (Fig. 5*A*). Similar results were observed when the effect of this inhibitor on 804G-ECM-induced NF-κB DNA binding activity was assessed by ELISA (not shown). Furthermore, Bay 11-7082 inhibited overexpression of IκBα mRNA induced by 804G-ECM, thus confirming that it inhibits the transcriptional activity of NF-κB (Fig. 8*B*). We have shown previously (3) that 804G-ECM induces spreading of the pancreatic beta cell, and this effect is glucose-dependent. NF-κB has been shown to be involved in cell motility through its effect on gene expression (reviewed in Ref. 29), leading us to hypothesize that 804G-ECM-induced NF-κB activity might be involved in spreading of beta cells by 804G-ECM. Treatment of cells with Bay 11-7082 significantly reduced spreading of cells on 804G-ECM as assessed after 24 h of culture (Fig. 5*B*).

In order to visualize actin cytoskeleton organization in cells after 24 h of culture, F-actin was labeled with phalloidin. As reported by our group (30), and as shown in Fig. 5*C*, cells cultured on 804G-ECM organize their actin to form distinct fibers in the cell interior, whereas no clear actin organization could be seen in cells plated on pLL. When treated with Bay 11-7082, the intracellular actin fibers were disrupted in cells cultured on 804G-ECM. Although evaluation of cortical actin organization can be hampered by the shape of a cell, and cells

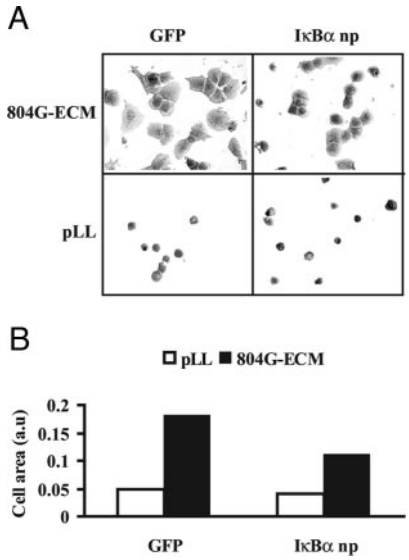

FIG. 6. **Adenovirus-mediated inhibition of NF-κB alters spreading of cells induced by 804G-ECM.** *A*, effect of nonphosphorylatable IκBα mutant (*IκBα np*) on cell spreading. Cells were infected with control virus (*GFP*) or with IκBαnp and plated on pLL- or on 804G-ECM-coated dishes. Cells were fixed 24 h later. *B*, the area of cells was quantified using ScionImage™ software. The data shown are representative of two independent experiments. *a.u.*, arbitrary units.

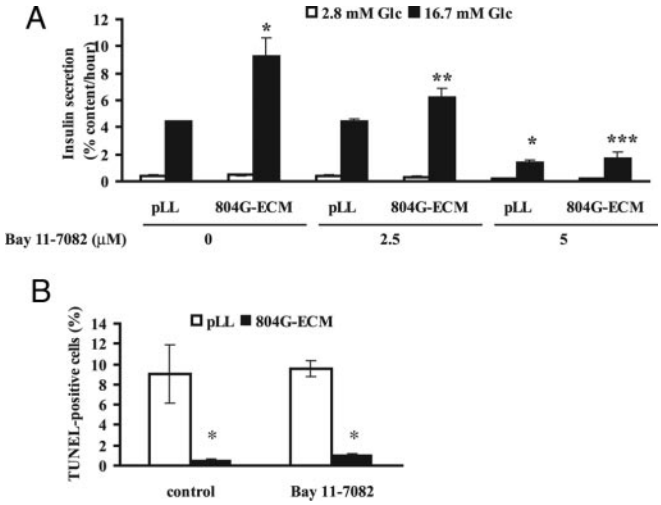

FIG. 7. **Inhibition of 804G-ECM-induced NF-κB activity affects glucose-stimulated insulin secretion but does not affect survival.** Cells were pretreated or not for 1 h with Bay 11-7082 before 24 h of culture on pLL or on 804G-ECM-coated dishes in the continued presence of the inhibitor. *A*, after the 24-h culture period, short term insulin secretion was measured over 1 h in static incubation at 2.8 and 16.7 mM glucose as indicated. For stimulated insulin secretion (16.7 mM glucose): *, $p < 0.01$ *versus* pLL control; **, $p < 0.02$ *versus* pLL 2.5 μM; ***, $p < 0.001$ *versus* 804G control ($n = 5$–6 from three independent experiments). *B*, cells were fixed 24 h later, and cellular death was quantified by TUNEL. *, $p < 0.05$ *versus* pLL in respective condition ($n = 3$).

on 804G-ECM in the presence of the inhibitor were less spread, close inspection of the confocal images (Fig. 5*C*) suggests a better developed and more consequent cortical actin ring in the treated cells.

Recombinant adenovirus expressing nondegradable IκBα (IκBαnp) has been shown to be an effective inhibitor of NF-κB nuclear translocation and transcriptional activity (19, 20). 804G-ECM-induced nuclear translocation of NF-κB was decreased in cells transduced with the recombinant IκBαnp virus as compared with cells transduced with the control (GFP) adenovirus (data not shown). The effect of IκBαnp on cell spreading was analyzed after 24 h of culture on pLL or on 804G-ECM. Spreading of cells infected with IκBαnp was reduced compared with cells infected with the control virus (Fig. 6), confirming that 804G-ECM-induced NF-κB activity is involved in the spreading of pancreatic beta cells induced by 804G-ECM.

Inhibition of NF-κB Activity; Effects on Insulin Secretion—The importance of cell-matrix interactions for optimal GSIS (insulin secreted at 16.7 mM glucose) by pancreatic beta cells in culture has been demonstrated by us (3) and by other groups (31–36). However, the mechanism involved has yet to be clarified. It has been suggested that there is a correlation between increased spreading of cells with their ability to respond optimally to glucose (3). As NF-κB activity seems to be involved in spreading of cells and organization of the actin cytoskeleton induced by 804G-ECM (Figs. 5 and 6), we hypothesized that it might be involved in the beneficial effects of 804G-ECM on GSIS. As shown in Fig. 7*A* and in Table I, GSIS of cells cultured for 24 h on 804G-ECM was significantly increased compared with cells cultured on pLL. Bay 11-7082 induced a dose-dependent decrease of GSIS of cells cultured on 804G-ECM, and a final concentration of 5 μM of this inhibitor induced a significant decrease of GSIS in cells on both pLL and 804G-ECM (Fig. 7*A* and Table I). Furthermore, the fold stimulation of insulin secretion (amount of insulin secreted at 16.7 mM glucose compared with that secreted at 2.8 mM glucose) was significantly decreased in treated cells, both on pLL and on 804G-ECM (Table I). In addition, Bay 11-7082 induced a significant decrease in "absolute" stimulated insulin secretion (*i.e.* amount of secreted insulin at 16.7 mM glucose, per dish and hour) as well as an increase in total insulin content of cells plated on 804G-ECM, as compared with the control condition (Table I). Cellular protein content was similar in all conditions (Table I). Bay 11-7082 thus inhibits insulin secretion. In summary, these results indicate that 804G-ECM-induced NF-κB activity is involved in spreading, actin cytoskeleton organization, and GSIS of pancreatic beta cells.

Inhibition of ECM-induced NF-κB Activity Does Not Affect Cell Survival—804G-ECM protects pancreatic beta cells against apoptosis (4), so we hypothesized that NF-κB might mediate its pro-survival effect. TUNEL assays were performed on cells cultured for 24 h on pLL or on 804G-ECM-coated dishes, in the absence or in the presence of Bay 11-7082. Treatment of cells with 5 μM Bay 11-7082 did not affect cell survival either on pLL or on 804G-ECM (Fig. 7*B*). Similar results were obtained using adenoviruses expressing nonphosphorylatable IκBα (data not shown). Therefore, it seems that 804G-ECM-induced activity of NF-κB is not involved in the control of survival and/or apoptosis of pancreatic beta cells.

Involvement of the MAP Kinase ERK Pathway in 804G-ECM-induced NF-κB Activity—We have shown previously that 804G-ECM induces phosphorylation of the MAP kinases ERK1 and ERK2, as well as of Akt/PKB, and that both pathways seemed to be involved in the anti-apoptotic effect of 804G-ECM (4). The signaling pathways involving ERK and Akt/PKB have both been reported to be able to activate NF-κB (5, 10, 29, 37). To get a preliminary insight into how 804G-ECM activates NF-κB transcriptional activity, the effects of PD98059 (inhibitor of MEK1 and thereby of the MAP kinase ERK pathway) and of LY294002 (inhibitor of PI3K and thereby of Akt/PKB signaling) on the DNA binding activity of NF-κB and on the overexpression of IκBα induced by 804G-ECM were analyzed. As shown in Fig. 8*A*, PD98059 inhibited the NF-κB DNA binding activity by 40% ($p < 0.0005$). We have shown previously that PD98059 inhibits the overexpression of IκBα induced by 804G-ECM (4). Taken together, these results suggest that the MAP kinase ERK pathway might be involved in the activation of NF-κB induced by 804G-ECM. By contrast, the inhibitor

TABLE I

Effect of inhibition of 804G-ECM-induced NF-κB activity on secretion and cell content of insulin

Cells were pretreated or not with 5 μM Bay 11-7082, plated on pLL- or 804G-ECM-coated dishes, and cultured for 24 h in the continued presence of this inhibitor. Fold increase indicates insulin secretion at 16.7 mM glucose/2.8 mM glucose.

	pLL		804G-ECM	
	Control	Bay 11-7082	Control	Bay 11-7082
Insulin secretion (16.7 mM Glc) (% content/h)	4.38 ± 0.06	1.36 ± 0.25[a]	9.23 ± 1.34[a]	1.71 ± 0.44[b]
Insulin secretion (2.8 mM Glc) (% content/h)	0.42 ± 0.04	0.21 ± 0.01[c]	0.47 ± 0.04	0.21 ± 0.025[c]
Fold increase	10.7 ± 1.7	6.1 ± 1.1[d]	19.7 ± 4.6	8 ± 1.6[d]
Insulin secretion (16.7 mM Glc) (ng/(dish × h))	14.96 ± 1.34	5.93 ± 1.01[d]	13.17 ± 2.58	4.42 ± 1.27[d]
Insulin secretion (2.8 mM Glc) (ng/(dish × h))	1.34 ± 0.2	0.97 ± 0.13	0.67 ± 0.08[e]	0.55 ± 0.07[f]
Insulin content (ng/dish)	325.8 ± 31	459.2 ± 51.9	141.5 ± 7.4[e]	276.5 ± 29.7[b]
Cellular protein content (μg/dish)	4.25 ± 0.38	4.42 ± 0.4	3.96 ± 0.39	4.56 ± 0.29

[a] $p < 0.01$ *versus* pLL control.
[b] $p < 0.002$ *versus* 804G-ECM control.
[c] $p < 0.005$ *versus* control condition on respective substrate.
[d] $p < 0.02$ *versus* control condition on respective substrate.
[e] $p < 0.02$ *versus* pLL control.
[f] $p < 0.02$ *versus* pLL Bay 11-7082 ($n = 5–6$ from three independent experiments). The cellular protein contents were measured in a separate series of experiments ($n = 3$ from two independent experiments).

LY294002 (50 μM) had no effect either on the DNA binding activity of NF-κB (Fig. 8*A*) or on the expression of IκBα (Fig. 8*B*), suggesting that the PI3K-Akt/PKB pathway is not involved in the regulation of the transcriptional activity of NF-κB by 804G-ECM.

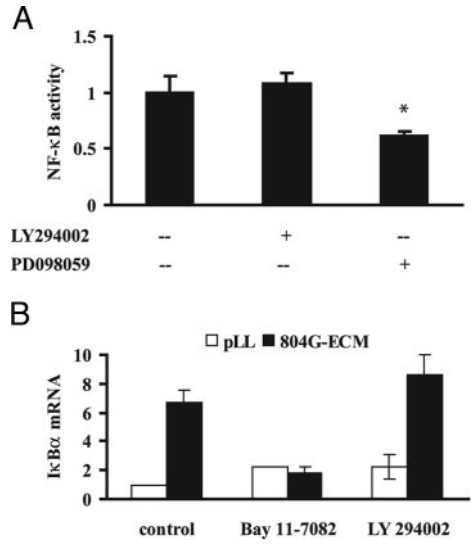

FIG. 8. *A*, effects of inhibitors of the MAP kinase ERK and the PI3K-Akt/PKB pathways (PD98059 and LY294002) on NF-κB DNA binding activity. Cells were pretreated or not with PD98059 (50 μM) or with LY294002 (50 μM) for 15–30 min before plating them on pLL- or on 804G-ECM-coated dishes. NF-κB DNA binding activity (normalized to control) was measured by ELISA (absorbance, 450–650 nm) after 2 h of exposure to 804G-ECM. *, $p < 0.0005$ ($n = 4$). *B*, effects of NF-κB inhibitor Bay 11-7082 and LY294002 on 804G-ECM-induced overexpression of IκBα mRNA. Cells were pretreated or not with Bay 11-7082 (5 μM) or with LY294002 (50 μM) for 15–30 min before plating them on pLL- or on 804G-ECM-coated dishes. mRNA was extracted after 4 h of culture, and IκBα mRNA levels were measured by reverse transcription-PCR. The results (means ± S.D. of two independent experiments) are shown as IκBα mRNA levels relative to the internal control L3, normalized to control condition (*pLL*, control).

DISCUSSION

In this report we show that 804G-ECM induces nuclear translocation of NF-κB, that it increases binding of NF-κB to DNA, and that it induces overexpression of the well established NF-κB target genes NF-κB1 (p105) and IκBα. Collectively, these data indicate that 804G-ECM induces NF-κB transcriptional activity in primary pancreatic beta cells. Others have reported that various ECMs are able to activate NF-κB in different cell types; however, most studies have been performed in cell lines (transformed or not), and to our knowledge there has been no such study in primary, nondividing cells.

In the canonical pathway leading to NF-κB activation, others have observed a substantial degradation of IκBα prior to NF-κB nuclear localization. In our system, the degradation of IκBα after 1 h of culture on 804G-ECM was not significant as compared with control (pLL), suggesting that only a small fraction of the total IκBα pool is degraded. The consequence would be that only a minor fraction of NF-κB cytosolic dimers is translocated to the nucleus. The fact that the cytoplasmic staining for NF-κB was still apparent in all cells exposed for 1 h to 804G-ECM sustains the latter hypothesis.

After 24 h of culture, IκBα protein levels were significantly increased on 804G-ECM as compared with pLL, and this increase was sustained for at least 48 h (4). This prolonged overexpression of IκBα is most interesting, because increased IκBα expression will both prevent additional NF-κB nuclear translocation and remove NF-κB from its nuclear binding sites (12) and could thus be responsible for the observed transience of 804G-ECM-induced NF-κB activity.

Several different signaling proteins have been reported to mediate signaling from ECM to NF-κB, including MAP kinase ERK (37), PI3K (5, 10), and Rho GTPase Rac (5, 10, 38). We have reported previously (4) that the MAP kinase-ERK and PI3K-Akt/PKB pathways are activated by 804G-ECM, suggesting that these latter pathways might be involved in 804G-ECM-induced NF-κB activity. Based on the use of selective inhibitors, we now show that the MAP kinase ERK pathway, but not the PI3K-Akt/PKB pathway, mediates the increased DNA binding activity of NF-κB as well as overexpression of IκBα induced by 804G-ECM.

As reported previously by our group, plating beta cells on 804G-ECM induces their attachment, cytoskeleton remodeling, and cell spreading. Here we report that long term blockage of NF-κB activity by pharmacological means (Bay 11-7082) or by adenoviral overexpression of nonphosphorylatable IκBα impairs 804G-ECM-induced spreading of the cells. This inhibitory effect might depend on expression of genes induced by NF-κB. Indeed, it has been reported by other groups that NF-κB can mediate overexpression of proteins involved in spreading and/or migration of cells (29).

Furthermore, we have observed that blocking NF-κB activity with Bay 11-7082 impairs GSIS, but it does not affect cell survival. While this work was in preparation, Norlin *et al.* (39) reported the effects of attenuating basal NF-κB activity on GSIS and on cell survival, using transgenic mice expressing nonphosphorylatable IκBα. Most interestingly, although their

approach was different from ours, the observed phenotypes because of NF-κB inhibition were similar in both models; GSIS was impaired, whereas beta cell survival was not affected.

We have shown previously that the MAP kinase ERK pathway could be involved in the pro-survival effect of the 804G-ECM but that it does not affect spreading of beta cells (4). As discussed above, this pathway seems to be involved in 804G-ECM induction of NF-κB activity as well. It is intriguing in this context that inhibition of NF-κB did not affect beta cell survival but that it did inhibit spreading of cells. ERK is known to affect the activities of a multitude of signaling pathways and transcription factors (40), regulating migration and survival of cells. Therefore, we propose that ERK effector(s) other than NF-κB might mediate its pro-survival effect. Spreading and/or migration of cells is an extremely complex process, involving the interplay of several signaling pathways (41). Further studies are therefore mandatory to better dissect the inter-dependence of the MAP kinase ERK and NF-κB pathways as well as other candidate signaling proteins (such as the Rho GTPases) involved in the effects of the 804G-ECM on the pancreatic beta cell.

What is the connection between NF-κB and GSIS? Different mechanisms may be involved, and in our opinion, they might well complete each other. One possible connection between NF-κB and GSIS is the actin cytoskeleton, and another one is regulation of gene expression. The actin filaments are organized in two ways in secretory cells as follows: a cortical F-actin web (rim or ring) underneath the plasma membrane and actin filament fibers distributed throughout the cytosol (42, 43). Many studies, including in pancreatic islets, have reported that the actin web limits the access of the secretory granules to the cell boundary; disruption or remodeling of the web is believed to be a prerequisite for exocytosis (42, 44–46). On the other hand, the function of actin filaments in the cell interior is less well understood. However, dynamic association of insulin-containing granules with actin cytoskeleton is thought to be involved in insulin exocytosis. As shown in this work, 804G-ECM induces remodeling of the actin cytoskeleton, leading to the appearance of actin filament fibers in the cell interior. Upon treatment with Bay 11-7082, these actin fibers were no more apparent, and this may underlie impaired insulin secretion. The cortical actin web appeared more prominent in the treated cells, and this may further impair insulin secretion. Therefore, we suggest that NF-κB might be involved in GSIS through the remodeling of the actin cytoskeleton. Depolarization-induced Ca^{2+} is a required step for GSIS to occur. Most interestingly, it has been reported that depolarization/Ca^{2+} influx can activate NF-κB transcriptional activity and that the ERK inhibitor PD98059 blocked this activation of NF-κB (47). However, the consequences of such depolarization-induced NF-κB activity are still unknown (47).

It has been shown that expression of genes implicated in glucose uptake, oxidative metabolism, and Ca^{2+}-triggered exocytosis is perturbed in transgenic mice expressing nonphosphorylatable IκBα (39). Furthermore, long term treatment of neuronal cells with tumor necrosis factor-α enhances depolarization-induced increases of Ca^{2+}, and it has been suggested that these effects are induced via altered gene expression mediated by NF-κB (48). Therefore, we propose that blockage of 804G-ECM-induced NF-κB activity might repress expression of genes necessary for well regulated GSIS.

It is now well acknowledged that NF-κB plays a major role in cytokine-induced beta cell dysfunction and apoptosis (17). However, this study shows a completely different aspect of NF-κB function in beta cells. A critical question arises: how does a given stimulus lead to specific biological end points through regulation of NF-κB activity? The functional consequences of NF-κB activity seem to depend on several different factors, such as the kinetics, extent, and context of activation of NF-κB. 804G-ECM-induced nuclear translocation of NF-κB appeared to be moderate when compared with that occurring after cytokine treatment. Furthermore, we show that 804G-ECM-induced binding of NF-κB to DNA declines after 2 h of exposure to 804G-ECM and that 804G-ECM-induced overexpression of IκBα and NF-κB1 (p105) mRNAs begins to decay after 8 h of culture, indicating that 804G-ECM-induced NF-κB activity is transient. This contrasts with the sustained activity of NF-κB induced by cytokines.[3] These differences in kinetics and extent of NF-κB activity may explain the different functional outcomes mediated by 804G-ECM- and cytokine-induced NF-κB activation. It has been reported that the first phase of IL-1β-induced NF-κB activity leads to the beneficial increase of beta cell defense/repair protein expression. By contrast, the second phase of NF-κB activity induced by IL-1β is harmful, because it leads to a sustained decrease of specific beta cell proteins like insulin, GLUT-2, and PDX-1 with a concomitant increase of other "aspecific" proteins and inducible nitric-oxide synthase transcription (49). In summary, these data combined with our own data support the concept that transient and/or low NF-κB activity is beneficial, whereas sustained and/or strong NF-κB activity is deleterious for the pancreatic beta cell.

Recently it has emerged that the transactivation potential of NF-κB is modulated by post-translational modifications, including phosphorylation and acetylation of NF-κB subunits as well as of histones surrounding NF-κB target genes (reviewed in Refs. 50 and 51). Site-specific phosphorylation of the p65 subunit of NF-κB can occur by a large variety of kinases in response to different stimuli (51) and may target NF-κB to a particular subset of genes (52). We hypothesize that IL-1β and 804G-ECM might induce phosphorylation of p65 on distinct sites, leading to different biological responses. Experiments aimed at exploring this hypothesis are underway in our laboratory. The next challenge would be to inhibit selectively the NF-κB activity leading to its deleterious effects without interfering with its beneficial outcomes.

The procedure leading to pancreatic beta cell isolation prior to pancreatic islet transplantation involves disruption of cell-cell and cell-ECM contacts (33, 53). The isolated pancreatic beta cells lose glucose responsiveness and eventually die when maintained in culture for a long period of time. By contrast, when layered on appropriate ECM, pancreatic islet cell function and survival can be maintained (3, 4, 31–33, 35, 36, 54). Here we show that ECM induces transient and moderate activity of the transcription factor NF-κB (p65) and that ECM-induced NF-κB activity is involved in spreading, cytoskeleton organization, and improved function (GSIS) of primary pancreatic beta cells. We propose that transient and moderate NF-κB activity is essential for well regulated glucose-stimulated insulin secretion in the pancreatic beta cell.

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REFERENCES

1. Miranti, C. K., and Brugge, J. S. (2002) *Nat. Cell Biol.* **4,** E83–E90
2. Juliano, R. L. (2002) *Annu. Rev. Pharmacol. Toxicol.* **42,** 283–323
3. Bosco, D., Meda, P., Halban, P. A., and Rouiller, D. G. (2000) *Diabetes* **49,** 233–243
4. Hammar, E., Parnaud, G., Bosco, D., Perriraz, N., Maedler, K., Donath, M., Rouiller, D. G., and Halban, P. A. (2004) *Diabetes* **53,** 2034–2041
5. Reyes-Reyes, M., Mora, N., Zentella, A., and Rosales, C. (2001) *J. Cell Sci.* **114,** 1579–1589
6. Rosales, C., and Juliano, R. (1996) *Cancer Res.* **56,** 2302–2305

[3] T. Mandrup-Poulsen, personal communication.

7. Lin, T. H., Rosales, C., Mondal, K., Bolen, J. B., Haskill, S., and Juliano, R. L. (1995) *J. Biol. Chem.* **270,** 16189–16197
8. Scatena, M., Almeida, M., Chaisson, M. L., Fausto, N., Nicosia, R. F., and Giachelli, C. M. (1998) *J. Cell Biol.* **141,** 1083–1093
9. Qwarnstrom, E. E., Ostberg, C. O., Turk, G. L., Richardson, C. A., and Bomsztyk, K. (1994) *J. Biol. Chem.* **269,** 30765–30768
10. Klein, S., de Fougerolles, A. R., Blaikie, P., Khan, L., Pepe, A., Green, C. D., Koteliansky, V., and Giancotti, F. G. (2002) *Mol. Cell. Biol.* **22,** 5912–5922
11. de Fougerolles, A. R., Chi-Rosso, G., Bajardi, A., Gotwals, P., Green, C. D., and Koteliansky, V. E. (2000) *Immunity* **13,** 749–758
12. Hayden, M. S., and Ghosh, S. (2004) *Genes Dev.* **18,** 2195–2224
13. Yamamoto, Y., and Gaynor, R. B. (2004) *Trends Biochem. Sci.* **29,** 72–79
14. Malek, S., Huxford, T., and Ghosh, G. (1998) *J. Biol. Chem.* **273,** 25427–25435
15. Brown, K., Park, S., Kanno, T., Franzoso, G., and Siebenlist, U. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 2532–2536
16. Scott, M. L., Fujita, T., Liou, H. C., Nolan, G. P., and Baltimore, D. (1993) *Genes Dev.* **7,** 1266–1276
17. Donath, M. Y., Storling, J., Maedler, K., and Mandrup-Poulsen, T. (2003) *J. Mol. Med.* **81,** 455–470
18. Rehman, K. K., Bertera, S., Bottino, R., Balamurugan, A. N., Mai, J. C., Mi, Z., Trucco, M., and Robbins, P. D. (2003) *J. Biol. Chem.* **278,** 9862–9868
19. Heimberg, H., Heremans, Y., Jobin, C., Leemans, R., Cardozo, A. K., Darville, M., and Eizirik, D. L. (2001) *Diabetes* **50,** 2219–2224
20. Giannoukakis, N., Rudert, W. A., Trucco, M., and Robbins, P. D. (2000) *J. Biol. Chem.* **275,** 36509–36513
21. Zeender, E., Maedler, K., Bosco, D., Berney, T., Donath, M. Y., and Halban, P. A. (2004) *J. Clin. Endocrinol. Metab.* **89,** 5059–5066
22. Rouiller, D. G., Cirulli, V., and Halban, P. A. (1990) *Exp. Cell Res.* **191,** 305–312
23. Schaffner, W., and Weissmann, C. (1973) *Anal. Biochem.* **56,** 502–514
24. Pierce, J. W., Schoenleber, R., Jesmok, G., Best, J., Moore, S. A., Collins, T., and Gerritsen, M. E. (1997) *J. Biol. Chem.* **272,** 21096–21103
25. Cardozo, A. K., Heimberg, H., Heremans, Y., Leeman, R., Kutlu, B., Kruhoffer, M., Orntoft, T., and Eizirik, D. L. (2001) *J. Biol. Chem.* **276,** 48879–48886
26. Herbert, V., Lau, K. S., Gottlieb, C. W., and Bleicher, S. J. (1965) *J. Clin. Endocrinol. Metab.* **25,** 1375–1384
27. Tian, B., and Brasier, A. R. (2003) *Recent Prog. Horm. Res.* **58,** 95–130
28. Thompson, J. E., Phillips, R. J., Erdjument-Bromage, H., Tempst, P., and Ghosh, S. (1995) *Cell* **80,** 573–582
29. Shishodia, S., and Aggarwal, B. B. (2004) *Biochem. Pharmacol.* **68,** 1071–1080
30. Parnaud, G., Hammar, E., Rouiller, D. G., and Bosco, D. (2005) *Am. J. Physiol.* **289,** E313–E321
31. Nagata, N., Gu, Y., Hori, H., Balamurugan, A. N., Touma, M., Kawakami, Y., Wang, W., Baba, T. T., Satake, A., Nozawa, M., Tabata, Y., and Inoue, K. (2001) *Cell Transplant* **10,** 447–451
32. Beattie, G. M., Lappi, D. A., Baird, A., and Hayek, A. (1991) *J. Clin. Endocrinol. Metab.* **73,** 93–98
33. Wang, R. N., and Rosenberg, L. (1999) *J. Endocrinol.* **163,** 181–190
34. Thomas, F. T., Contreras, J. L., Bilbao, G., Ricordi, C., Curiel, D., and Thomas, J. M. (1999) *Surgery* **126,** 299–304
35. Kaiser, N., Corcos, A. P., Tur-Sinai, A., Ariav, Y., and Cerasi, E. (1988) *Endocrinology* **123,** 834–840
36. Kaido, T., Yebra, M., Cirulli, V., and Montgomery, A. M. (2004) *J. Biol. Chem.* **279,** 53762–53769
37. McGilvray, I. D., Lu, Z., Bitar, R., Dackiw, A. P., Davreux, C. J., and Rotstein, O. D. (1997) *J. Biol. Chem.* **272,** 10287–10294
38. Zahir, N., Lakins, J. N., Russell, A., Ming, W., Chatterjee, C., Rozenberg, G. I., Marinkovich, M. P., and Weaver, V. M. (2003) *J. Cell Biol.* **163,** 1397–1407
39. Norlin, S., Ahlgren, U., and Edlund, H. (2005) *Diabetes* **54,** 125–132
40. Howe, A. K., Aplin, A. E., and Juliano, R. L. (2002) *Curr. Opin. Genet. Dev.* **12,** 30–35
41. Alahari, S. K., Reddig, P. J., and Juliano, R. L. (2002) *Int. Rev. Cytol.* **220,** 145–184
42. Orci, L., Gabbay, K. H., and Malaisse, W. J. (1972) *Science* **175,** 1128–1130
43. Gabbiani, G., Malaisse-Lagae, F., Blondel, B., and Orci, L. (1974) *Endocrinology* **95,** 1630–1635
44. Li, G., Rungger-Brandle, E., Just, I., Jonas, J. C., Aktories, K., and Wollheim, C. B. (1994) *Mol. Biol. Cell* **5,** 1199–1213
45. Nevins, A. K., and Thurmond, D. C. (2003) *Am. J. Physiol.* **285,** C698–C710
46. Thurmond, D. C., Gonelle-Gispert, C., Furukawa, M., Halban, P. A., and Pessin, J. E. (2003) *Mol. Endocrinol.* **17,** 732–742
47. Bernal-Mizrachi, E., Wen, W., Shornick, M., and Permutt, M. A. (2002) *Diabetes* **51,** Suppl. 3, 484–488
48. Furukawa, K., and Mattson, M. P. (1998) *J. Neurochem.* **70,** 1876–1886
49. Papaccio, G., Graziano, A., d'Aquino, R., Valiante, S., and Naro, F. (2005) *J. Cell Physiol.* **204,** 124–130
50. Chen, L. F., and Greene, W. C. (2004) *Nat. Rev. Mol. Cell. Biol.* **5,** 392–401
51. Vermeulen, L., De Wilde, G., Notebaert, S., Vanden Berghe, W., and Haegeman, G. (2002) *Biochem. Pharmacol.* **64,** 963–970
52. Anrather, J., Racchumi, G., and Iadecola, C. (2005) *J. Biol. Chem.* **280,** 244–252
53. Rosenberg, L., Wang, R., Paraskevas, S., and Maysinger, D. (1999) *Surgery* **126,** 393–398
54. Ris, F., Hammar, E., Bosco, D., Pilloud, C., Maedler, K., Donath, M. Y., Oberholzer, J., Zeender, E., Morel, P., Rouiller, D., and Halban, P. A. (2002) *Diabetologia* **45,** 841–850

4. Major findings and perspectives of Part III (Paper 2)

In this second part of the thesis work, the major aims were to establish whether 804G-ECM is able to activate the transcription factor NF-κB, and to investigate the function(s) of 804G-ECM-induced NF-κB activity in the pancreatic beta cell.

As expected from the gene expression analyses (described in Part III, § 2), 804G-ECM does induce nuclear translocation of NF-κB (p65), its binding to DNA, as well as its transcriptional activity leading to the increased expression of IκBα. We observed that the effect of 804G-ECM on NF-κB nuclear translocation and DNA-binding activity is weak as compared to the effect induced by the cytokine IL-1β. Furthermore, DNA-binding activity of NF-κB and over-expression of IκBα induced by 804G-ECM are transient (as assessed up to 24h of culture).

To get an insight into how 804G-ECM activates NF-κB, we investigated whether blockade of the MAP kinase ERK and of the PI3K-Akt pathways would affect the 804G-ECM-induced NF-κB activity. We found that blockade of the MAP kinase ERK pathway but not of the PI3K-Akt pathway reduced DNA-binding activity of NF-κB as well as 804G-ECM-induced IκBα overexpression. It is difficult to reconcile these data with the fact that blockade of the MAP kinase ERK pathway inhibits the pro-survival effect of the 804G-ECM while that of the NF-κB pathway does not. Probably, another effector of the ERK pathway might mediate the pro-survival effect of the 804G-ECM, and this warrants further investigation.

As described in the introduction, NF-κB is known to play either anti- or pro-apoptotic roles, and it has been reported that ECM-induced NF-κB activity can play an anti-apoptotic role. Therefore, the finding that 804G-ECM-induced NF-κB activity seems *not* to be involved in the pro-survival effect of the 804G-ECM was unexpected. By contrast, NF-κB appears to be involved in 804G-ECM-induced actin cytoskeleton organization and cell spreading, and glucose-stimulated insulin secretion. It has been reported by others that NF-κB induces expression of genes that are involved in spreading and/or migration of cells, such as chemokines (218; 234). As 804G-ECM induces overexpression of chemokines, it will be interesting to assess whether this is involved in its effects on actin cytoskeleton organization and/or spreading. In addition, we hypothesize that actin cytoskeleton organization and regulation of gene expression are both at least in part involved in the function of 804G-ECM-induced NF-κB activity in glucose-stimulated insulin secretion.

Our finding that 804G-ECM-induced NF-κB activity is involved in the function of the pancreatic beta cell is supported by a recent study using transgenic mice (291). This study reports that beta cell-specific expression of a non-phosphorylable IκBα mutant (that inhibits NF-κB) leads to deficient glucose-stimulated insulin secretion (291). Our data showing that 804G-ECM-induced NF-κB activity is involved in glucose-stimulated insulin secretion lead to a number of new questions. The most important ones are the following:

- Which is the NF-κB subunit involved? Reducing the expression of specific NF-κB subunits (the p65 subunit for example) using the RNAi technology should provide an answer to this question. In addition, this approach would confirm the results obtained with the NF-κB inhibitor Bay 11-7082. Indeed, it is important to keep in mind that this pharmacological inhibitor blocks NF-κB activity indirectly via inhibition of IκBα phosphorylation and subsequent degradation.

- How does ECM-induced NF-κB activity regulate cell spreading, actin cytoskeleton organization and glucose-stimulated insulin secretion? Is this regulation due to direct effects (via protein phosphorylation and/or acetylation, …) or due to altered gene expression?

- Which are the signaling pathways leading to ECM-induced NF-κB activation? How is the ERK signaling pathway involved? What is the role of Ca^{2+}? Indeed, it has been shown that Ca^{2+} plays a primordial role in glucose-stimulated insulin secretion and Ca^{2+} has been reported to be involved in the activation of NF-κB.

- Is the ECM which surrounds the islets *in vivo* providing signals leading to basal NF-κB activity? It has been proposed that beta cells do have a basal NF-κB activity *in vivo*, which allows these cells to function normally (291). It is tempting to hypothesize that this activity is at least in part provided by the surrounding ECM.

- Why are the biological outcomes of IL-1β-induced and ECM-induced NF-κB activities so different? Several hypotheses might explain those differences:

1) The temporal pattern and intensity of IL-1β and ECM-induced NF-κB activities are different. This might lead NF-κB to target a different subset of genes. It would be interesting to perform a Chromatin immunoprecipitation (CHIP) experiment to confirm this hypothesis.

2) Alternatively, IL-1β and/or ECM might induce phosphorylation and/or acetylation of NF-κB subunits on different sites, leading to different patterns of gene expression. Part of the gene expression specificity may also be codetermined by the surrounding chromatin modifications, as well as by the surrounding transcription cofactors.

3) Finally, NF-κB has been proposed to play a yet uncharacterized role in the cytoplasm, which might be involved in the effects of the ECM on cell spreading and cytoskeleton organization. This hypothesis merits further investigation.

In conclusion, we have found that 804G-ECM induces an NF-κB activity that is involved in 804G-ECM-induced actin cytoskeleton organization and cell spreading, as well as glucose-stimulated insulin secretion in pancreatic beta cells. However, the mechanisms involved in these effects of 804G-ECM-induced NF-κB activity remain to be unveiled.

PART IV : SUMMARY AND GENERAL DISCUSSION

It is now generally accepted that cell death by apoptosis is involved in the pathogenesis of type 1 but also of type 2 diabetes. In addition, the pancreatic beta cell seems to be particularly sensitive towards apoptotic insults (as discussed in Part I, § 1.2.2.). Therefore, a better understanding of mechanisms regulating survival of the pancreatic beta cell is a major topic in diabetes research.

Anoikis, apoptosis induced by detachment from or by interaction of cells with non-adapted ECM, is now a well accepted concept. However, it has been proposed that detachment-induced apoptosis in different cell types and consecutive to different biological events is mediated by entirely different mechanisms. Therefore, much work remains to be done to establish the mechanistic linkages between absent and/or deficient cell-ECM adhesion and anoikis for each given cell-type and/or context.

ECM has been shown to play a crucial role in several biological functions, including cell migration, survival and regulation of gene expression. The role of ECM in the protection of cells against apoptotic insults is now generally acknowledged for a number of cell types. Prior to the beginning of this thesis work, there had been some reports indicating that ECM may also play an important role in pancreatic beta cell survival and function, but those data remained to be confirmed. The ECM protein laminin has been detected in the pancreatic islet of Langerhans. Previous work in our laboratory has shown that rat pancreatic beta cells express the laminin-5 receptors integrins $\alpha 3\beta 1$ and $\alpha 6\beta 1$. Furthermore, the 804G-ECM, that contains laminin-5, induces spreading of beta cells and improves glucose-stimulated insulin secretion, indicating that this ECM might interact with the integrins expressed on rat beta cells. The 804G-ECM was therefore chosen as a model for this thesis work, in order to investigate the following points:

- the role of ECM in the survival of pancreatic beta cells

- the signaling pathways activated by ECM and their involvement in the pro-survival effect of ECM

- the effect of ECM on gene expression and signaling involved

In our first paper, we show that 804G-ECM improves survival of beta cells, and that interactions between laminin-5 and $\beta 1$ integrins are involved in this improved survival induced by 804G-ECM. Caspase-8 activity is reduced in cells cultured on 804G-ECM as compared to cells on cultured on the control substrate pLL. 804G-ECM induces a rapid phosphorylation of FAK, reinforcing the hypothesis that it activates integrins expressed on beta cells. 804G-ECM also induces phosphorylation of the signaling proteins Akt and ERK. Blockage of the PI3K-Akt pathway (using the PI3K inhibitor LY294002) and of the MAP kinase ERK pathway (using the MEK1 inhibitor PD98059) both increase cell death on 804G-ECM after 4h of culture, while only the ERK pathway inhibitor was found to inhibit the long-term (48h) pro-survival effect of 804G-

ECM. Finally, 804G-ECM was found to induce the overexpression of one NF-κB target gene: IκBα.

In our second paper, the central hypothesis was that 804G-ECM induces the transcriptional activity of the transcription factor NF-κB, and that 804G-ECM-induced NF-κB activity might be involved in some of the effects of 804G-ECM on the pancreatic beta cell. We found that 804G-ECM induces weak and transient NF-κB DNA-binding and transcriptional activities. Furthermore, we found that blockage of NF-κB activity reduces 804G-ECM-induced spreading and cytoskeleton organization, inhibits glucose-stimulated insulin secretion, while it has no effect on cell survival. This however does not exclude that 804G-ECM might activate other transcription factors, leading to altered gene expression that could underlie its anti-apoptotic effect. Finally, we found that blockage of the ERK pathway reduces both the DNA-binding and transcriptional activities of NF-κB.

There are some additional questions raised by these studies that merit further investigation. First, the studies performed with the 804G-ECM do not exclude that there might exist other ECMs that are better adapted for the rat pancreatic beta cell to function and survive optimally. In addition, it remains possible that the 804G-ECM might activate other signaling pathways that have an even more important role in the pancreatic beta cell survival than the PI3K-Akt and MAP kinase ERK pathways. Furthermore, we cannot exclude that insulin may play a role in the observed effects of 804G-ECM on the pancreatic beta cell. An autocrine role for insulin on the pancreatic beta cell function and survival has been reported, although this concept is still controversial. Previous work performed in our laboratory has shown that exposure of pancreatic beta cells to 804G-ECM enhances glucose-stimulated insulin secretion. Signaling pathways activated by growth factors and insulin are often very similar to those activated by ECM. In addition, engagement of integrins to cognate ECM has been shown to cooperate with insulin signaling pathways to protect cells from apoptosis. Therefore, and particularly since the experiments were performed in stimulatory glucose concentrations (11.2 mM glucose), we cannot exclude that the increased insulin secretion by cells on 804G-ECM as compared to cells on pLL may contribute to the pro-survival effect of 804G-ECM as well as to induction of signaling pathways by 804G-ECM. A possible effect of insulin on the effects of 804G-ECM on the pancreatic beta cell thus warrants further investigation.

Based on the fact that the MAP kinase ERK pathway can mediate integrin-mediated overexpression of anti-apoptotic genes such as c-FLIP or Bcl-2, we hypothesized that 804G-ECM may induce overexpression of these genes. FLIP was considered as a particularly interesting candidate, as it has been reported to inhibit caspase-8, which activity is decreased in cells cultured on 804G-ECM. However, preliminary studies indicate that 804G-ECM does not induce overexpression of either c-FLIP or Bcl-2. Therefore, it remains to be assessed whether the long-term anti-apoptotic effect of ECM-induced ERK activation is related to altered gene expression or whether other mechanisms are involved.

Although the major findings and perspectives relative to Paper 1 and Paper 2 have been discussed in the previous sections, several points relative to this thesis work deserve additional discussion. First of all, why did we choose to use *exclusively* primary pancreatic beta cells? The difficulty of isolating the pancreatic beta cells, and therefore the difficulty to obtain enough material to perform biochemical experiments have led many groups to use transformed beta cell lines (that are often derived from insulinomas developed in transgenic mice expressing the SV40 T gene). Although some of these cell lines have been very useful for a large number of studies, and although they retain some major features of the normal beta cell, they are not always good substitutes for primary beta cells. This appears especially obvious in the context of studies assessing the role of ECM on beta cell survival and ECM-induced intracellular signaling, because the transformed cell lines are almost by definition anchorage-independent. In addition, each cell type expresses its own set of integrins leading it to interact with specific ECM proteins, and this is well recognized to be influenced by transformation. Furthermore, the signaling proteins activated by a given ECM and the hierarchy among these signaling pathways seem to differ between primary and transformed cells. Indeed, when the effect of 804G-ECM on FAK and Akt phosphorylation was investigated in the cell line INS-1E derived from rat insulinoma, it was obvious that phosphorylation of these proteins in response to 804G-ECM was considerably different as compared to the primary beta cells. In addition, short-term exposure (1 h) of INS-1E cells to 804G-ECM did not affect DNA-binding activity of NF-κB. Therefore, we chose to perform all experiments with primary beta cells. Nevertheless, the exclusive use of primary cells has hampered us to go deeper into the activation of intra-cellular signaling pathways by 804G-ECM. For example, studies using pharmacological inhibitors need further confirmation by biochemical experiments (such as transfection of cells with dominant-negative and/or dominant-positive Akt mutants, for example). Such experiments remain very difficult to perform on primary beta cells.

We observed that phosphorylation of the signaling proteins FAK, Akt and ERK induced by 804G-ECM is very weak as compared to that induced by growth factors, for example. It remains to be established whether an ECM that is “perfectly” adapted to the rat pancreatic beta cells (such as the one that naturally surrounds the islets in *vivo*, for example) would induce a greater increase in protein phosphorylation, or whether ECMs in general induce weak phosphorylation of these proteins as compared to growth factors in primary beta cells. In this context, it is interesting to note that others have reported that the ECM protein fibronectin induces a very weak phosphorylation of ERK in fibroblasts as compared to growth factors (170). This leads to the following question: is there truly a correlation between the intensity of activation of a given signaling pathway and its (positive) effect on the final biological outcome? In several different contexts, it has been proposed that a moderate and well-controlled activity of a given signaling pathway leads to a physiological effect, while constitutive or dysregulated activation of a signaling cascade can lead to deleterious effects that ultimately lead to serious illnesses such as cancer. If one views signaling cascades that coexist in the cell as an integrated network of pathways in a given place and at a given time-point in the cell, one might

speculate that a delicate balance exists between these pathways. Therefore, it appears that a moderate and controlled activation, in contrast to an exaggerated activation, of a given pathway or transcription factor will not disrupt the balance of signaling pathways in the cell. The intensity of a given stimulus in determining the biological outcomes of a given signaling pathway is therefore emerging as an important parameter to be taken in account. Much research is based on experiments performed using transient or stable overexpression of proteins. However, the physiological relevance of the observed effects induced by overexpression of proteins (in particular constitutive active mutants of kinases, for examples) is uncertain. Indeed, the observed effects induced by massive overexpression might be caused, at least in part, by non-specific disruption of the balance coexisting between different signaling pathways.

It is now quite clear that different signaling pathways do not exist independently of each other, but communicate, interact and cooperate in a synergistic way to promote cell survival. In addition to the nature and the intensity of a given stimuli, other important parameters that influence the endpoint of a given signaling pathway are emerging: the concept of space (the three-dimensional space inside the cell) and of time (duration and pulsatility of the signal). It has been proposed by Frisch and colleagues that the architectural state of the cytoskeleton can affect the interactions between signaling molecules in the three-dimensional space (56). For example, the endpoint of the ERK pathway has been shown to be affected by the state of the actin cytoskeleton. The state of the cytoskeleton is a parameter in addition to the regulation of gene expression that may explain the observed differences in the relative importance of the PI3K-Akt and ERK pathways in the protection of the beta cell against apoptosis in the short (4h) and long-term (48h) experiments (Paper 1). After 4h of culture, cells are only beginning to spread, while after 48h of culture they are completely spread, with a well-defined actin cytoskeleton on 804G-ECM. Therefore, it is tempting to speculate that the state of the cytoskeleton as well as the level of expression of 804G-ECM-induced proteins at a given time-point are two factors that determine the importance of the PI3K-Akt and MAP kinase ERK pathways for pancreatic beta cell survival. The duration of a given signal now also appears to be crucial for the biological consequences of a given signaling pathway. This has been proposed for both the ERK pathway and the transcription factor NF-κB. Indeed, both ERK and NF-κB have been shown to be able to play contrasting roles (pro-apoptotic and anti-apoptotic roles, for example), and recent papers propose that it is the duration and/or the pulsatility of the stimuli inducing their activity that determine the ultimate fate of the cell.

The findings reported in our second paper have led us to conclude that 804G-ECM-induced NF-κB activity is beneficial for pancreatic beta cell function, at least in part because it is moderate and transient. Our results contrast with previous findings reporting that NF-κB plays deleterious roles in beta cells, leading to their dysfunction and death. In the context of diabetes, blocking NF-κB activity is considered by some researchers as a most promising therapeutic means to improve beta cell survival in order to prevent the onset of diabetes. However, these

studies have neglected the fact that the nature, intensity and/or duration of the inducing stimuli can affect the biological outcomes of NF-κB activity and have focused exclusively on NF-κB activity that is induced by long-term (at least 48h) treatment with high doses of cytokines. Our data showing that NF-κB might be essential for the pancreatic beta cell to function normally is supported by a recent study using transgenic mice expressing non-phosphorylatable IκBα (291). We therefore hope that these data showing a new facet of NF-κB function in the pancreatic beta cell will force some investigators to be cautious in initiating clinical studies aimed at blocking NF-κB activity in diabetic patients.

A possible general role for ECM in the protection of islets against diverse insults leading to beta cell dysfunction and/or apoptosis and consequent loss of beta cell mass leading ultimately to a diabetic state *in vivo* remains to be unraveled. Studies using conditional knock-out mice harboring beta cell-specific deletion of specific ECM proteins (such as laminin-5) and/or of specific integrins (such as β1 integrin) should provide clues for a possible general role of ECM in the prevention against diabetes. However, data from such transgenic models will need to be interpreted with caution given the possibility of compensation by parallel, possibly redundant, pathways.

It has been reported that high glucose concentrations can modify expression of integrins, leading to altered cell-matrix interactions (292; 293). Furthermore, it has also been proposed that hyperglycemia can induce modifications (by nonenzymatic glycosylation) of ECM proteins that also results in modified cell-ECM interactions (294; 295). It is therefore tempting to hypothesize that altered cell-matrix interactions induced by hyperglycemia might lead to reduced protection of beta cells against pro-apoptotic stimuli. This could further accelerate the reduction of beta cell mass which is involved in the pathogenesis of diabetes. Therefore, it would be interesting to assess whether there is an alteration of the islet ECM and/or a modification of the beta cell integrin expression during the pathogenesis of diabetes. One animal model that could be used to verify this hypothesis is diabetes-prone mice fed with high-fat diet.

In conclusion, the findings of this thesis work demonstrate the importance of ECM for the pancreatic beta cell survival, as well as for the regulation of gene expression and the activity of kinases. ECM should therefore be viewed as a crucial part of the islet microenvironment that could provide protection of beta cells against apoptotic insults that are involved in the pathogenesis of diabetes.

REFERENCES

1. Wang RN, Paraskevas S, Rosenberg L: Characterization of integrin expression in islets isolated from hamster, canine, porcine, and human pancreas. *J Histochem Cytochem* 47:499-506, 1999
2. van Deijnen JH, Hulstaert CE, Wolters GH, van Schilfgaarde R: Significance of the peri-insular extracellular matrix for islet isolation from the pancreas of rat, dog, pig, and man. *Cell Tissue Res* 267:139-146, 1992
3. Lang J: Molecular mechanisms and regulation of insulin exocytosis as a paradigm of endocrine secretion. *Eur J Biochem* 259:3-17, 1999
4. Bosco D, Orci L, Meda P: Homologous but not heterologous contact increases the insulin secretion of individual pancreatic B-cells. *Exp Cell Res* 184:72-80, 1989
5. Bosco D, Meda P, Halban PA, Rouiller DG: Importance of cell-matrix interactions in rat islet beta-cell secretion in vitro: role of alpha6beta1 integrin. *Diabetes* 49:233-243, 2000
6. Halban PA: Cellular sources of new pancreatic beta cells and therapeutic implications for regenerative medicine. *Nat Cell Biol* 6:1021-1025, 2004
7. Bonner-Weir S: Life and death of the pancreatic beta cells. *Trends Endocrinol Metab* 11:375-378, 2000
8. Rhodes CJ: Type 2 diabetes-a matter of beta-cell life and death? *Science* 307:380-384, 2005
9. Dickson LM, Rhodes CJ: Pancreatic beta-cell growth and survival in the onset of type 2 diabetes: a role for protein kinase B in the Akt? *Am J Physiol Endocrinol Metab* 287:E192-198, 2004
10. Atkinson MA, Eisenbarth GS: Type 1 diabetes: new perspectives on disease pathogenesis and treatment. *Lancet* 358:221-229, 2001
11. Kahn SE: Engineering a new beta-cell: a critical venture requiring special attention to constantly changing physiological needs. *Semin Cell Dev Biol* 15:359-370, 2004
12. Donath MY, Halban PA: Decreased beta-cell mass in diabetes: significance, mechanisms and therapeutic implications. *Diabetologia* 47:581-589, 2004
13. Masiello P: Animal models of type 2 diabetes with reduced pancreatic beta cell mass. *The International Journal of Biochemistry and Cell Biology In press*, 2005
14. Danial NN, Korsmeyer SJ: Cell death: critical control points. *Cell* 116:205-219, 2004
15. Strasser A, O'Connor L, Dixit VM: Apoptosis signaling. *Annu Rev Biochem* 69:217-245, 2000
16. Kerr JF, Wyllie AH, Currie AR: Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br J Cancer* 26:239-257, 1972
17. Mandrup-Poulsen T: Apoptotic signal transduction pathways in diabetes. *Biochem Pharmacol* 66:1433-1440, 2003
18. Mathis D, Vence L, Benoist C: beta-Cell death during progression to diabetes. *Nature* 414:792-798, 2001
19. Eizirik DL, Mandrup-Poulsen T: A choice of death--the signal-transduction of immune-mediated beta-cell apoptosis. *Diabetologia* 44:2115-2133, 2001
20. Maedler K, Spinas GA, Lehmann R, Sergeev P, Weber M, Fontana A, Kaiser N, Donath MY: Glucose induces beta-cell apoptosis via upregulation of the Fas receptor in human islets. *Diabetes* 50:1683-1690, 2001
21. Donath MY, Gross DJ, Cerasi E, Kaiser N: Hyperglycemia-induced beta-cell apoptosis in pancreatic islets of Psammomys obesus during development of diabetes. *Diabetes* 48:738-744, 1999
22. Pick A, Clark J, Kubstrup C, Levisetti M, Pugh W, Bonner-Weir S, Polonsky KS: Role of apoptosis in failure of beta-cell mass compensation for insulin resistance and beta-cell defects in the male Zucker diabetic fatty rat. *Diabetes* 47:358-364, 1998
23. Farilla L, Hui H, Bertolotto C, Kang E, Bulotta A, Di Mario U, Perfetti R: Glucagon-like peptide-1 promotes islet cell growth and inhibits apoptosis in Zucker diabetic rats. *Endocrinology* 143:4397-4408, 2002
24. Sakuraba H, Mizukami H, Yagihashi N, Wada R, Hanyu C, Yagihashi S: Reduced beta-cell mass and expression of oxidative stress-related DNA damage in the islet of Japanese Type II diabetic patients. *Diabetologia* 45:85-96, 2002
25. Butler AE, Janson J, Bonner-Weir S, Ritzel R, Rizza RA, Butler PC: Beta-cell deficit and increased beta-cell apoptosis in humans with type 2 diabetes. *Diabetes* 52:102-110, 2003
26. O'Brien BA, Fieldus WE, Field CJ, Finegood DT: Clearance of apoptotic beta-cells is reduced in neonatal autoimmune diabetes-prone rats. *Cell Death Differ* 9:457-464, 2002

27. El-Assaad W, Buteau J, Peyot ML, Nolan C, Roduit R, Hardy S, Joly E, Dbaibo G, Rosenberg L, Prentki M: Saturated fatty acids synergize with elevated glucose to cause pancreatic beta-cell death. *Endocrinology* 144:4154-4163, 2003
28. Kahn SE: Clinical review 135: The importance of beta-cell failure in the development and progression of type 2 diabetes. *J Clin Endocrinol Metab* 86:4047-4058, 2001
29. Mandrup-Poulsen T: beta-cell apoptosis: stimuli and signaling. *Diabetes* 50 Suppl 1:S58-63, 2001
30. Hui H, Dotta F, Di Mario U, Perfetti R: Role of caspases in the regulation of apoptotic pancreatic islet beta-cells death. *J Cell Physiol* 200:177-200, 2004
31. Oyadomari S, Araki E, Mori M: Endoplasmic reticulum stress-mediated apoptosis in pancreatic beta-cells. *Apoptosis* 7:335-345, 2002
32. Donath MY, Storling J, Maedler K, Mandrup-Poulsen T: Inflammatory mediators and islet beta-cell failure: a link between type 1 and type 2 diabetes. *J Mol Med* 81:455-470, 2003
33. Bosman FT, Stamenkovic I: Functional structure and composition of the extracellular matrix. *J Pathol* 200:423-428, 2003
34. Hynes RO: Integrins: bidirectional, allosteric signaling machines. *Cell* 110:673-687, 2002
35. Juliano RL: Signal transduction by cell adhesion receptors and the cytoskeleton: functions of integrins, cadherins, selectins, and immunoglobulin-superfamily members. *Annu Rev Pharmacol Toxicol* 42:283-323, 2002
36. Giancotti FG, Ruoslahti E: Integrin signaling. *Science* 285:1028-1032., 1999
37. Lee JW, Juliano R: Mitogenic signal transduction by integrin- and growth factor receptor-mediated pathways. *Mol Cells* 17:188-202, 2004
38. Wehrle-Haller B, Imhof BA: Integrin-dependent pathologies. *J Pathol* 200:481-487, 2003
39. Engbring JA, Kleinman HK: The basement membrane matrix in malignancy. *J Pathol* 200:465-470, 2003
40. Gilbert RE, Cox A, Dziadek M, Cooper ME, Jerums G: Extracellular matrix and its interactions in the diabetic kidney: a molecular biological approach. *J Diabetes Complications* 9:252-254, 1995
41. Gumbiner BM: Cell adhesion: the molecular basis of tissue architecture and morphogenesis. *Cell* 84:345-357, 1996
42. Alberts: *Molecular Biology of the Cell*. London-New York, 1994
43. Burridge K, Fath K, Kelly T, Nuckolls G, Turner C: Focal adhesions: transmembrane junctions between the extracellular matrix and the cytoskeleton. *Annu Rev Cell Biol* 4:487-525, 1988
44. Geiger B, Bershadsky A, Pankov R, Yamada KM: Transmembrane crosstalk between the extracellular matrix--cytoskeleton crosstalk. *Nat Rev Mol Cell Biol* 2:793-805, 2001
45. Danen EH, Sonnenberg A: Integrins in regulation of tissue development and function. *J Pathol* 201:632-641, 2003
46. Bouvard D, Brakebusch C, Gustafsson E, Aszodi A, Bengtsson T, Berna A, Fassler R: Functional consequences of integrin gene mutations in mice. *Circ Res* 89:211-223, 2001
47. Schwartz MA, Ginsberg MH: Networks and crosstalk: integrin signalling spreads. *Nat Cell Biol* 4:E65-68, 2002
48. Damsky CH, Ilic D: Integrin signaling: it's where the action is. *Curr Opin Cell Biol* 14:594-602, 2002
49. Bagutti C, Wobus AM, Fassler R, Watt FM: Differentiation of embryonal stem cells into keratinocytes: comparison of wild-type and beta 1 integrin-deficient cells. *Dev Biol* 179:184-196, 1996
50. Fassler R, Pfaff M, Murphy J, Noegel AA, Johansson S, Timpl R, Albrecht R: Lack of beta 1 integrin gene in embryonic stem cells affects morphology, adhesion, and migration but not integration into the inner cell mass of blastocysts. *J Cell Biol* 128:979-988, 1995
51. Carroll JM, Romero MR, Watt FM: Suprabasal integrin expression in the epidermis of transgenic mice results in developmental defects and a phenotype resembling psoriasis. *Cell* 83:957-968, 1995
52. Howlett AR, Bailey N, Damsky C, Petersen OW, Bissell MJ: Cellular growth and survival are mediated by beta 1 integrins in normal human breast epithelium but not in breast carcinoma. *J Cell Sci* 108 (Pt 5):1945-1957, 1995
53. Meredith JE, Jr., Fazeli B, Schwartz MA: The extracellular matrix as a cell survival factor. *Mol Biol Cell* 4:953-961, 1993
54. Kantengwa S, Baetens D, Sadoul K, Buck CA, Halban PA, Rouiller DG: Identification and characterization of alpha 3 beta 1 integrin on primary and transformed rat islet cells. *Exp Cell Res* 237:394-402, 1997

55. Frisch SM, Francis H: Disruption of epithelial cell-matrix interactions induces apoptosis. *J Cell Biol* 124:619-626, 1994
56. Frisch SM, Screaton RA: Anoikis mechanisms. *Curr Opin Cell Biol* 13:555-562., 2001
57. Thomas FT, Contreras JL, Bilbao G, Ricordi C, Curiel D, Thomas JM: Anoikis, extracellular matrix, and apoptosis factors in isolated cell transplantation. *Surgery* 126:299-304, 1999
58. Wang RN, Rosenberg L: Maintenance of beta-cell function and survival following islet isolation requires re-establishment of the islet-matrix relationship. *J Endocrinol* 163:181-190., 1999
59. Boudreau N, Sympson CJ, Werb Z, Bissell MJ: Suppression of ICE and apoptosis in mammary epithelial cells by extracellular matrix. *Science* 267:891-893, 1995
60. Ris F, Hammar E, Bosco D, Pilloud C, Maedler K, Donath MY, Oberholzer J, Zeender E, Morel P, Rouiller D, Halban PA: Impact of integrin-matrix matching and inhibition of apoptosis on the survival of purified human beta-cells in vitro. *Diabetologia* 45:841-850, 2002
61. Riedl SJ, Shi Y: Molecular mechanisms of caspase regulation during apoptosis. *Nat Rev Mol Cell Biol* 5:897-907, 2004
62. Aoudjit F, Vuori K: Matrix attachment regulates Fas-induced apoptosis in endothelial cells: a role for c-flip and implications for anoikis. *J Cell Biol* 152:633-643, 2001
63. Frisch SM: Evidence for a function of death-receptor-related, death-domain-containing proteins in anoikis. *Curr Biol* 9:1047-1049, 1999
64. Rytomaa M, Martins LM, Downward J: Involvement of FADD and caspase-8 signalling in detachment-induced apoptosis. *Curr Biol* 9:1043-1046, 1999
65. Stupack DG, Puente XS, Boutsaboualoy S, Storgard CM, Cheresh DA: Apoptosis of adherent cells by recruitment of caspase-8 to unligated integrins. *J Cell Biol* 155:459-470, 2001
66. Stupack DG, Cheresh DA: Get a ligand, get a life: integrins, signaling and cell survival. *J Cell Sci* 115:3729-3738, 2002
67. Raff MC: Social controls on cell survival and cell death. *Nature* 356:397-400, 1992
68. Pullan S, Wilson J, Metcalfe A, Edwards GM, Goberdhan N, Tilly J, Hickman JA, Dive C, Streuli CH: Requirement of basement membrane for the suppression of programmed cell death in mammary epithelium. *J Cell Sci* 109 (Pt 3):631-642, 1996
69. Farrelly N, Lee YJ, Oliver J, Dive C, Streuli CH: Extracellular matrix regulates apoptosis in mammary epithelium through a control on insulin signaling. *J Cell Biol* 144:1337-1348, 1999
70. Manohar A, Shome SG, Lamar J, Stirling L, Iyer V, Pumiglia K, DiPersio CM: Alpha 3 beta 1 integrin promotes keratinocyte cell survival through activation of a MEK/ERK signaling pathway. *J Cell Sci* 117:4043-4054, 2004
71. Zhang Z, Vuori K, Reed JC, Ruoslahti E: The alpha 5 beta 1 integrin supports survival of cells on fibronectin and up-regulates Bcl-2 expression. *Proc Natl Acad Sci U S A* 92:6161-6165, 1995
72. Lee JW, Juliano RL: alpha5beta1 integrin protects intestinal epithelial cells from apoptosis through a phosphatidylinositol 3-kinase and protein kinase B-dependent pathway. *Mol Biol Cell* 11:1973-1987, 2000
73. Gu J, Fujibayashi A, Yamada KM, Sekiguchi K: Laminin-10/11 and fibronectin differentially prevent apoptosis induced by serum removal via phosphatidylinositol 3-kinase/Akt- and MEK1/ERK-dependent pathways. *J Biol Chem* 277:19922-19928, 2002
74. Barberis L, Wary KK, Fiucci G, Liu F, Hirsch E, Brancaccio M, Altruda F, Tarone G, Giancotti FG: Distinct roles of the adaptor protein Shc and focal adhesion kinase in integrin signaling to ERK. *J Biol Chem* 275:36532-36540, 2000
75. Hirsch E, Barberis L, Brancaccio M, Azzolino O, Xu D, Kyriakis JM, Silengo L, Giancotti FG, Tarone G, Fassler R, Altruda F: Defective Rac-mediated proliferation and survival after targeted mutation of the beta1 integrin cytodomain. *J Cell Biol* 157:481-492., 2002
76. Rousselle P, Aumailley M: Kalinin is more efficient than laminin in promoting adhesion of primary keratinocytes and some other epithelial cells and has a different requirement for integrin receptors. *J Cell Biol* 125:205-214, 1994
77. Carter WG, Ryan MC, Gahr PJ: Epiligrin, a new cell adhesion ligand for integrin alpha 3 beta 1 in epithelial basement membranes. *Cell* 65:599-610, 1991
78. Ricordi C: Islet transplantation: a brave new world. *Diabetes* 52:1595-1603, 2003
79. Rosenberg L, Wang R, Paraskevas S, Maysinger D: Structural and functional changes resulting from islet isolation lead to islet cell death. *Surgery* 126:393-398, 1999
80. Beattie GM, Lappi DA, Baird A, Hayek A: Functional impact of attachment and purification in the short term culture of human pancreatic islets. *J Clin Endocrinol Metab* 73:93-98, 1991

81. Nagata N, Gu Y, Hori H, Balamurugan AN, Touma M, Kawakami Y, Wang W, Baba TT, Satake A, Nozawa M, Tabata Y, Inoue K: Evaluation of insulin secretion of isolated rat islets cultured in extracellular matrix. *Cell Transplant* 10:447-451, 2001
82. Hulinsky I, Harrington J, Cooney S, Silink M: Insulin secretion and DNA synthesis of cultured islets of Langerhans are influenced by the matrix. *Pancreas* 11:309-314, 1995
83. Kaiser N, Corcos AP, Tur-Sinai A, Ariav Y, Cerasi E: Monolayer culture of adult rat pancreatic islets on extracellular matrix: long term maintenance of differentiated B-cell function. *Endocrinology* 123:834-840, 1988
84. Langhofer M, Hopkinson SB, Jones JC: The matrix secreted by 804G cells contains laminin-related components that participate in hemidesmosome assembly in vitro. *J Cell Sci* 105 (Pt 3):753-764, 1993
85. Hormia M, Falk-Marzillier J, Plopper G, Tamura RN, Jones JC, Quaranta V: Rapid spreading and mature hemidesmosome formation in HaCaT keratinocytes induced by incubation with soluble laminin-5r. *J Invest Dermatol* 105:557-561, 1995
86. Baker SE, DiPasquale AP, Stock EL, Quaranta V, Fitchmun M, Jones JC: Morphogenetic effects of soluble laminin-5 on cultured epithelial cells and tissue explants. *Exp Cell Res* 228:262-270, 1996
87. Hayek A, Beattie GM, Cirulli V, Lopez AD, Ricordi C, Rubin JS: Growth factor/matrix-induced proliferation of human adult beta-cells. *Diabetes* 44:1458-1460., 1995
88. Miranti CK, Brugge JS: Sensing the environment: a historical perspective on integrin signal transduction. *Nat Cell Biol* 4:E83-90, 2002
89. Kornberg L, Earp HS, Parsons JT, Schaller M, Juliano RL: Cell adhesion or integrin clustering increases phosphorylation of a focal adhesion-associated tyrosine kinase. *J Biol Chem* 267:23439-23442, 1992
90. Hanks SK, Calalb MB, Harper MC, Patel SK: Focal adhesion protein-tyrosine kinase phosphorylated in response to cell attachment to fibronectin. *Proc Natl Acad Sci U S A* 89:8487-8491, 1992
91. Schaller MD, Borgman CA, Cobb BS, Vines RR, Reynolds AB, Parsons JT: pp125FAK a structurally distinctive protein-tyrosine kinase associated with focal adhesions. *Proc Natl Acad Sci U S A* 89:5192-5196, 1992
92. Parsons JT: Focal adhesion kinase: the first ten years. *J Cell Sci* 116:1409-1416, 2003
93. Schwartz MA: Integrin signaling revisited. *Trends Cell Biol* 11:466-470., 2001
94. Howe AK, Aplin AE, Juliano RL: Anchorage-dependent ERK signaling--mechanisms and consequences. *Curr Opin Genet Dev* 12:30-35., 2002
95. Hanks SK, Ryzhova L, Shin NY, Brabek J: Focal adhesion kinase signaling activities and their implications in the control of cell survival and motility. *Front Biosci* 8:d982-996, 2003
96. Guan JL, Trevithick JE, Hynes RO: Fibronectin/integrin interaction induces tyrosine phosphorylation of a 120-kDa protein. *Cell Regul* 2:951-964, 1991
97. Guan JL, Shalloway D: Regulation of focal adhesion-associated protein tyrosine kinase by both cellular adhesion and oncogenic transformation. *Nature* 358:690-692, 1992
98. Burridge K, Turner CE, Romer LH: Tyrosine phosphorylation of paxillin and pp125FAK accompanies cell adhesion to extracellular matrix: a role in cytoskeletal assembly. *J Cell Biol* 119:893-903, 1992
99. George EL, Georges-Labouesse EN, Patel-King RS, Rayburn H, Hynes RO: Defects in mesoderm, neural tube and vascular development in mouse embryos lacking fibronectin. *Development* 119:1079-1091, 1993
100. Ilic D, Furuta Y, Kanazawa S, Takeda N, Sobue K, Nakatsuji N, Nomura S, Fujimoto J, Okada M, Yamamoto T: Reduced cell motility and enhanced focal adhesion contact formation in cells from FAK-deficient mice. *Nature* 377:539-544, 1995
101. Calalb MB, Polte TR, Hanks SK: Tyrosine phosphorylation of focal adhesion kinase at sites in the catalytic domain regulates kinase activity: a role for Src family kinases. *Mol Cell Biol* 15:954-963, 1995
102. Schlaepfer DD, Hanks SK, Hunter T, van der Geer P: Integrin-mediated signal transduction linked to Ras pathway by GRB2 binding to focal adhesion kinase. *Nature* 372:786-791, 1994
103. Calalb MB, Zhang X, Polte TR, Hanks SK: Focal adhesion kinase tyrosine-861 is a major site of phosphorylation by Src. *Biochem Biophys Res Commun* 228:662-668, 1996
104. Schaller MD, Hildebrand JD, Shannon JD, Fox JW, Vines RR, Parsons JT: Autophosphorylation of the focal adhesion kinase, pp125FAK, directs SH2-dependent binding of pp60src. *Mol Cell Biol* 14:1680-1688, 1994

105. Lipfert L, Haimovich B, Schaller MD, Cobb BS, Parsons JT, Brugge JS: Integrin-dependent phosphorylation and activation of the protein tyrosine kinase pp125FAK in platelets. *J Cell Biol* 119:905-912, 1992
106. Cary LA, Guan JL: Focal adhesion kinase in integrin-mediated signaling. *Front Biosci* 4:D102-113, 1999
107. Songyang Z, Shoelson SE, Chaudhuri M, Gish G, Pawson T, Haser WG, King F, Roberts T, Ratnofsky S, Lechleider RJ, et al.: SH2 domains recognize specific phosphopeptide sequences. *Cell* 72:767-778, 1993
108. Chen HC, Appeddu PA, Isoda H, Guan JL: Phosphorylation of tyrosine 397 in focal adhesion kinase is required for binding phosphatidylinositol 3-kinase. *J Biol Chem* 271:26329-26334, 1996
109. Chen Q, Kinch MS, Lin TH, Burridge K, Juliano RL: Integrin-mediated cell adhesion activates mitogen-activated protein kinases. *J Biol Chem* 269:26602-26605, 1994
110. Akagi T, Murata K, Shishido T, Hanafusa H: v-Crk activates the phosphoinositide 3-kinase/AKT pathway by utilizing focal adhesion kinase and H-Ras. *Mol Cell Biol* 22:7015-7023, 2002
111. Schlaepfer DD, Hunter T: Evidence for in vivo phosphorylation of the Grb2 SH2-domain binding site on focal adhesion kinase by Src-family protein-tyrosine kinases. *Mol Cell Biol* 16:5623-5633, 1996
112. Gilmore AP, Romer LH: Inhibition of focal adhesion kinase (FAK) signaling in focal adhesions decreases cell motility and proliferation. *Mol Biol Cell* 7:1209-1224, 1996
113. Oktay M, Wary KK, Dans M, Birge RB, Giancotti FG: Integrin-mediated activation of focal adhesion kinase is required for signaling to Jun NH2-terminal kinase and progression through the G1 phase of the cell cycle. *J Cell Biol* 145:1461-1469, 1999
114. Zhao JH, Reiske H, Guan JL: Regulation of the cell cycle by focal adhesion kinase. *J Cell Biol* 143:1997-2008, 1998
115. Cary LA, Chang JF, Guan JL: Stimulation of cell migration by overexpression of focal adhesion kinase and its association with Src and Fyn. *J Cell Sci* 109 (Pt 7):1787-1794, 1996
116. Owen JD, Ruest PJ, Fry DW, Hanks SK: Induced focal adhesion kinase (FAK) expression in FAK-null cells enhances cell spreading and migration requiring both auto- and activation loop phosphorylation sites and inhibits adhesion-dependent tyrosine phosphorylation of Pyk2. *Mol Cell Biol* 19:4806-4818, 1999
117. Sieg DJ, Hauck CR, Schlaepfer DD: Required role of focal adhesion kinase (FAK) for integrin-stimulated cell migration. *J Cell Sci* 112 (Pt 16):2677-2691, 1999
118. Richardson A, Malik RK, Hildebrand JD, Parsons JT: Inhibition of cell spreading by expression of the C-terminal domain of focal adhesion kinase (FAK) is rescued by coexpression of Src or catalytically inactive FAK: a role for paxillin tyrosine phosphorylation. *Mol Cell Biol* 17:6906-6914, 1997
119. Frisch SM, Vuori K, Ruoslahti E, Chan-Hui PY: Control of adhesion-dependent cell survival by focal adhesion kinase. *J Cell Biol* 134:793-799, 1996
120. Xu LH, Owens LV, Sturge GC, Yang X, Liu ET, Craven RJ, Cance WG: Attenuation of the expression of the focal adhesion kinase induces apoptosis in tumor cells. *Cell Growth Differ* 7:413-418, 1996
121. Hungerford JE, Compton MT, Matter ML, Hoffstrom BG, Otey CA: Inhibition of pp125FAK in cultured fibroblasts results in apoptosis. *J Cell Biol* 135:1383-1390, 1996
122. Ilic D, Almeida EA, Schlaepfer DD, Dazin P, Aizawa S, Damsky CH: Extracellular matrix survival signals transduced by focal adhesion kinase suppress p53-mediated apoptosis. *J Cell Biol* 143:547-560, 1998
123. Almeida EA, Ilic D, Han Q, Hauck CR, Jin F, Kawakatsu H, Schlaepfer DD, Damsky CH: Matrix survival signaling: from fibronectin via focal adhesion kinase to c-Jun NH(2)-terminal kinase. *J Cell Biol* 149:741-754, 2000
124. Gilmore AP, Metcalfe AD, Romer LH, Streuli CH: Integrin-mediated survival signals regulate the apoptotic function of Bax through its conformation and subcellular localization. *J Cell Biol* 149:431-446, 2000
125. Sonoda Y, Matsumoto Y, Funakoshi M, Yamamoto D, Hanks SK, Kasahara T: Anti-apoptotic role of focal adhesion kinase (FAK). Induction of inhibitor-of-apoptosis proteins and apoptosis suppression by the overexpression of FAK in a human leukemic cell line, HL-60. *J Biol Chem* 275:16309-16315, 2000

126. Chan PC, Lai JF, Cheng CH, Tang MJ, Chiu CC, Chen HC: Suppression of ultraviolet irradiation-induced apoptosis by overexpression of focal adhesion kinase in Madin-Darby canine kidney cells. *J Biol Chem* 274:26901-26906, 1999
127. Escobedo JA, Navankasattusas S, Kavanaugh WM, Milfay D, Fried VA, Williams LT: cDNA cloning of a novel 85 kd protein that has SH2 domains and regulates binding of PI3-kinase to the PDGF beta-receptor. *Cell* 65:75-82, 1991
128. Skolnik EY, Margolis B, Mohammadi M, Lowenstein E, Fischer R, Drepps A, Ullrich A, Schlessinger J: Cloning of PI3 kinase-associated p85 utilizing a novel method for expression/cloning of target proteins for receptor tyrosine kinases. *Cell* 65:83-90, 1991
129. Kapeller R, Cantley LC: Phosphatidylinositol 3-kinase. *Bioessays* 16:565-576, 1994
130. Cantley LC: The phosphoinositide 3-kinase pathway. *Science* 296:1655-1657, 2002
131. Cantrell DA: Phosphoinositide 3-kinase signalling pathways. *J Cell Sci* 114:1439-1445, 2001
132. Rameh LE, Arvidsson A, Carraway KL, 3rd, Couvillon AD, Rathbun G, Crompton A, VanRenterghem B, Czech MP, Ravichandran KS, Burakoff SJ, Wang DS, Chen CS, Cantley LC: A comparative analysis of the phosphoinositide binding specificity of pleckstrin homology domains. *J Biol Chem* 272:22059-22066, 1997
133. Franke TF, Yang SI, Chan TO, Datta K, Kazlauskas A, Morrison DK, Kaplan DR, Tsichlis PN: The protein kinase encoded by the Akt proto-oncogene is a target of the PDGF-activated phosphatidylinositol 3-kinase. *Cell* 81:727-736, 1995
134. Lawlor MA, Alessi DR: PKB/Akt: a key mediator of cell proliferation, survival and insulin responses? *J Cell Sci* 114:2903-2910, 2001
135. Downward J: PI 3-kinase, Akt and cell survival. *Semin Cell Dev Biol* 15:177-182, 2004
136. Bellacosa A, Testa JR, Staal SP, Tsichlis PN: A retroviral oncogene, akt, encoding a serine-threonine kinase containing an SH2-like region. *Science* 254:274-277, 1991
137. Coffer PJ, Woodgett JR: Molecular cloning and characterisation of a novel putative protein-serine kinase related to the cAMP-dependent and protein kinase C families. *Eur J Biochem* 201:475-481, 1991
138. Jones PF, Jakubowicz T, Pitossi FJ, Maurer F, Hemmings BA: Molecular cloning and identification of a serine/threonine protein kinase of the second-messenger subfamily. *Proc Natl Acad Sci U S A* 88:4171-4175, 1991
139. Datta SR, Brunet A, Greenberg ME: Cellular survival: a play in three Akts. *Genes Dev* 13:2905-2927, 1999
140. Jones PF, Jakubowicz T, Hemmings BA: Molecular cloning of a second form of rac protein kinase. *Cell Regul* 2:1001-1009, 1991
141. Konishi H, Shinomura T, Kuroda S, Ono Y, Kikkawa U: Molecular cloning of rat RAC protein kinase alpha and beta and their association with protein kinase C zeta. *Biochem Biophys Res Commun* 205:817-825, 1994
142. Konishi H, Kuroda S, Tanaka M, Matsuzaki H, Ono Y, Kameyama K, Haga T, Kikkawa U: Molecular cloning and characterization of a new member of the RAC protein kinase family: association of the pleckstrin homology domain of three types of RAC protein kinase with protein kinase C subspecies and beta gamma subunits of G proteins. *Biochem Biophys Res Commun* 216:526-534, 1995
143. Kohn AD, Takeuchi F, Roth RA: Akt, a pleckstrin homology domain containing kinase, is activated primarily by phosphorylation. *J Biol Chem* 271:21920-21926, 1996
144. Burgering BM, Coffer PJ: Protein kinase B (c-Akt) in phosphatidylinositol-3-OH kinase signal transduction. *Nature* 376:599-602, 1995
145. Alessi DR, Andjelkovic M, Caudwell B, Cron P, Morrice N, Cohen P, Hemmings BA: Mechanism of activation of protein kinase B by insulin and IGF-1. *Embo J* 15:6541-6551, 1996
146. James SR, Downes CP, Gigg R, Grove SJ, Holmes AB, Alessi DR: Specific binding of the Akt-1 protein kinase to phosphatidylinositol 3,4,5-trisphosphate without subsequent activation. *Biochem J* 315 (Pt 3):709-713, 1996
147. Alessi DR, James SR, Downes CP, Holmes AB, Gaffney PR, Reese CB, Cohen P: Characterization of a 3-phosphoinositide-dependent protein kinase which phosphorylates and activates protein kinase Balpha. *Curr Biol* 7:261-269, 1997
148. Brazil DP, Yang ZZ, Hemmings BA: Advances in protein kinase B signalling: AKTion on multiple fronts. *Trends Biochem Sci* 29:233-242, 2004
149. Troussard AA, Mawji NM, Ong C, Mui A, St -Arnaud R, Dedhar S: Conditional knock-out of integrin-linked kinase demonstrates an essential role in protein kinase B/Akt activation. *J Biol Chem* 278:22374-22378, 2003

150. Walker EH, Pacold ME, Perisic O, Stephens L, Hawkins PT, Wymann MP, Williams RL: Structural determinants of phosphoinositide 3-kinase inhibition by wortmannin, LY294002, quercetin, myricetin, and staurosporine. *Mol Cell* 6:909-919, 2000
151. Chen HC, Guan JL: Association of focal adhesion kinase with its potential substrate phosphatidylinositol 3-kinase. *Proc Natl Acad Sci U S A* 91:10148-10152, 1994
152. King WG, Mattaliano MD, Chan TO, Tsichlis PN, Brugge JS: Phosphatidylinositol 3-kinase is required for integrin-stimulated AKT and Raf-1/mitogen-activated protein kinase pathway activation. *Mol Cell Biol* 17:4406-4418, 1997
153. Yao R, Cooper GM: Requirement for phosphatidylinositol-3 kinase in the prevention of apoptosis by nerve growth factor. *Science* 267:2003-2006, 1995
154. Cho H, Thorvaldsen JL, Chu Q, Feng F, Birnbaum MJ: Akt1/PKBalpha is required for normal growth but dispensable for maintenance of glucose homeostasis in mice. *J Biol Chem* 276:38349-38352, 2001
155. Yang ZZ, Tschopp O, Baudry A, Dummler B, Hynx D, Hemmings BA: Physiological functions of protein kinase B/Akt. *Biochem Soc Trans* 32:350-354, 2004
156. Zha J, Harada H, Yang E, Jockel J, Korsmeyer SJ: Serine phosphorylation of death agonist BAD in response to survival factor results in binding to 14-3-3 not BCL-X(L). *Cell* 87:619-628, 1996
157. Datta SR, Dudek H, Tao X, Masters S, Fu H, Gotoh Y, Greenberg ME: Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. *Cell* 91:231-241, 1997
158. del Peso L, Gonzalez-Garcia M, Page C, Herrera R, Nunez G: Interleukin-3-induced phosphorylation of BAD through the protein kinase Akt. *Science* 278:687-689, 1997
159. Madrid LV, Wang CY, Guttridge DC, Schottelius AJ, Baldwin AS, Jr., Mayo MW: Akt suppresses apoptosis by stimulating the transactivation potential of the RelA/p65 subunit of NF-kappaB. *Mol Cell Biol* 20:1626-1638, 2000
160. Khwaja A: Akt is more than just a Bad kinase. *Nature* 401:33-34, 1999
161. Khwaja A, Rodriguez-Viciana P, Wennstrom S, Warne PH, Downward J: Matrix adhesion and Ras transformation both activate a phosphoinositide 3-OH kinase and protein kinase B/Akt cellular survival pathway. *Embo J* 16:2783-2793, 1997
162. Gary DS, Mattson MP: Integrin signaling via the PI3-kinase-Akt pathway increases neuronal resistance to glutamate-induced apoptosis. *J Neurochem* 76:1485-1496., 2001
163. Le Gall M, Chambard JC, Breittmayer JP, Grall D, Pouyssegur J, Van Obberghen-Schilling E: The p42/p44 MAP kinase pathway prevents apoptosis induced by anchorage and serum removal. *Mol Biol Cell* 11:1103-1112, 2000
164. Peyssonnaux C, Eychene A: The Raf/MEK/ERK pathway: new concepts of activation. *Biol Cell* 93:53-62, 2001
165. Roux PP, Blenis J: ERK and p38 MAPK-activated protein kinases: a family of protein kinases with diverse biological functions. *Microbiol Mol Biol Rev* 68:320-344, 2004
166. Wada T, Penninger JM: Mitogen-activated protein kinases in apoptosis regulation. *Oncogene* 23:2838-2849, 2004
167. Schlessinger J: Cell signaling by receptor tyrosine kinases. *Cell* 103:211-225, 2000
168. Juliano RL, Reddig P, Alahari S, Edin M, Howe A, Aplin A: Integrin regulation of cell signalling and motility. *Biochem Soc Trans* 32:443-446, 2004
169. Pearson G, Robinson F, Beers Gibson T, Xu BE, Karandikar M, Berman K, Cobb MH: Mitogen-activated protein (MAP) kinase pathways: regulation and physiological functions. *Endocr Rev* 22:153-183, 2001
170. Zhu X, Assoian RK: Integrin-dependent activation of MAP kinase: a link to shape-dependent cell proliferation. *Mol Biol Cell* 6:273-282, 1995
171. Morino N, Mimura T, Hamasaki K, Tobe K, Ueki K, Kikuchi K, Takehara K, Kadowaki T, Yazaki Y, Nojima Y: Matrix/integrin interaction activates the mitogen-activated protein kinase, p44erk-1 and p42erk-2. *J Biol Chem* 270:269-273, 1995
172. Mainiero F, Murgia C, Wary KK, Curatola AM, Pepe A, Blumemberg M, Westwick JK, Der CJ, Giancotti FG: The coupling of alpha6beta4 integrin to Ras-MAP kinase pathways mediated by Shc controls keratinocyte proliferation. *Embo J* 16:2365-2375, 1997
173. Schlaepfer DD, Hunter T: Focal adhesion kinase overexpression enhances ras-dependent integrin signaling to ERK2/mitogen-activated protein kinase through interactions with and activation of c-Src. *J Biol Chem* 272:13189-13195, 1997
174. Schlaepfer DD, Jones KC, Hunter T: Multiple Grb2-mediated integrin-stimulated signaling pathways to ERK2/mitogen-activated protein kinase: summation of both c-Src- and focal adhesion kinase-initiated tyrosine phosphorylation events. *Mol Cell Biol* 18:2571-2585, 1998

175. Reyes-Reyes M, Mora N, Zentella A, Rosales C: Phosphatidylinositol 3-kinase mediates integrin-dependent NF-kappaB and MAPK activation through separate signaling pathways. *J Cell Sci* 114:1579-1589, 2001
176. Danilkovitch A, Donley S, Skeel A, Leonard EJ: Two independent signaling pathways mediate the antiapoptotic action of macrophage-stimulating protein on epithelial cells. *Mol Cell Biol* 20:2218-2227, 2000
177. Giroux S, Tremblay M, Bernard D, Cardin-Girard JF, Aubry S, Larouche L, Rousseau S, Huot J, Landry J, Jeannotte L, Charron J: Embryonic death of Mek1-deficient mice reveals a role for this kinase in angiogenesis in the labyrinthine region of the placenta. *Curr Biol* 9:369-372, 1999
178. Pages G, Guerin S, Grall D, Bonino F, Smith A, Anjuere F, Auberger P, Pouyssegur J: Defective thymocyte maturation in p44 MAP kinase (Erk 1) knockout mice. *Science* 286:1374-1377, 1999
179. Cheung EC, Slack RS: Emerging role for ERK as a key regulator of neuronal apoptosis. *Sci STKE* 2004:PE45, 2004
180. Rul W, Zugasti O, Roux P, Peyssonnaux C, Eychene A, Franke TF, Lenormand P, Fort P, Hibner U: Activation of ERK, controlled by Rac1 and Cdc42 via Akt, is required for anoikis. *Ann N Y Acad Sci* 973:145-148, 2002
181. Ajenjo N, Canon E, Sanchez-Perez I, Matallanas D, Leon J, Perona R, Crespo P: Subcellular localization determines the protective effects of activated ERK2 against distinct apoptogenic stimuli in myeloid leukemia cells. *J Biol Chem* 279:32813-32823, 2004
182. Jost M, Huggett TM, Kari C, Rodeck U: Matrix-independent survival of human keratinocytes through an EGF receptor/MAPK-kinase-dependent pathway. *Mol Biol Cell* 12:1519-1527, 2001
183. McFall A, Ulku A, Lambert QT, Kusa A, Rogers-Graham K, Der CJ: Oncogenic Ras blocks anoikis by activation of a novel effector pathway independent of phosphatidylinositol 3-kinase. *Mol Cell Biol* 21:5488-5499, 2001
184. Zahir N, Lakins JN, Russell A, Ming W, Chatterjee C, Rozenberg GI, Marinkovich MP, Weaver VM: Autocrine laminin-5 ligates alpha6beta4 integrin and activates RAC and NFkappaB to mediate anchorage-independent survival of mammary tumors. *J Cell Biol* 163:1397-1407, 2003
185. Zugasti O, Rul W, Roux P, Peyssonnaux C, Eychene A, Franke TF, Fort P, Hibner U: Raf-MEK-Erk cascade in anoikis is controlled by Rac1 and Cdc42 via Akt. *Mol Cell Biol* 21:6706-6717, 2001
186. Holst LS, Mulder H, Manganiello V, Sundler F, Ahren B, Holm C, Degerman E: Protein kinase B is expressed in pancreatic beta cells and activated upon stimulation with insulin-like growth factor I. *Biochem Biophys Res Commun* 250:181-186, 1998
187. Srinivasan S, Bernal-Mizrachi E, Ohsugi M, Permutt MA: Glucose promotes pancreatic islet beta-cell survival through a PI 3-kinase/Akt-signaling pathway. *Am J Physiol Endocrinol Metab* 283:E784-793, 2002
188. Aikin R, Rosenberg L, Maysinger D: Phosphatidylinositol 3-kinase signaling to Akt mediates survival in isolated canine islets of Langerhans. *Biochem Biophys Res Commun* 277:455-461, 2000
189. Liu W, Chin-Chance C, Lee EJ, Lowe WL, Jr.: Activation of phosphatidylinositol 3-kinase contributes to insulin-like growth factor I-mediated inhibition of pancreatic beta-cell death. *Endocrinology* 143:3802-3812, 2002
190. Buteau J, El-Assaad W, Rhodes CJ, Rosenberg L, Joly E, Prentki M: Glucagon-like peptide-1 prevents beta cell glucolipotoxicity. *Diabetologia* 47:806-815, 2004
191. Wang Q, Li L, Xu E, Wong V, Rhodes C, Brubaker PL: Glucagon-like peptide-1 regulates proliferation and apoptosis via activation of protein kinase B in pancreatic INS-1 beta cells. *Diabetologia* 47:478-487, 2004
192. Wrede CE, Dickson LM, Lingohr MK, Briaud I, Rhodes CJ: Protein kinase B/Akt prevents fatty acid-induced apoptosis in pancreatic beta-cells (INS-1). *J Biol Chem* 277:49676-49684, 2002
193. Tuttle RL, Gill NS, Pugh W, Lee JP, Koeberlein B, Furth EE, Polonsky KS, Naji A, Birnbaum MJ: Regulation of pancreatic beta-cell growth and survival by the serine/threonine protein kinase Akt1/PKBalpha. *Nat Med* 7:1133-1137, 2001
194. Bernal-Mizrachi E, Wen W, Stahlhut S, Welling CM, Permutt MA: Islet beta cell expression of constitutively active Akt1/PKB alpha induces striking hypertrophy, hyperplasia, and hyperinsulinemia. *J Clin Invest* 108:1631-1638, 2001
195. Gannon M, Shiota C, Postic C, Wright CV, Magnuson M: Analysis of the Cre-mediated recombination driven by rat insulin promoter in embryonic and adult mouse pancreas. *Genesis* 26:139-142, 2000

196. Bernal-Mizrachi E, Fatrai S, Johnson JD, Ohsugi M, Otani K, Han Z, Polonsky KS, Permutt MA: Defective insulin secretion and increased susceptibility to experimental diabetes are induced by reduced Akt activity in pancreatic islet beta cells. *J Clin Invest* 114:928-936, 2004
197. Larsen CM, Wadt KA, Juhl LF, Andersen HU, Karlsen AE, Su MS, Seedorf K, Shapiro L, Dinarello CA, Mandrup-Poulsen T: Interleukin-1beta-induced rat pancreatic islet nitric oxide synthesis requires both the p38 and extracellular signal-regulated kinase 1/2 mitogen-activated protein kinases. *J Biol Chem* 273:15294-15300, 1998
198. Ammendrup A, Maillard A, Nielsen K, Aabenhus Andersen N, Serup P, Dragsbaek Madsen O, Mandrup-Poulsen T, Bonny C: The c-Jun amino-terminal kinase pathway is preferentially activated by interleukin-1 and controls apoptosis in differentiating pancreatic beta-cells. *Diabetes* 49:1468-1476, 2000
199. Pavlovic D, Andersen NA, Mandrup-Poulsen T, Eizirik DL: Activation of extracellular signal-regulated kinase (ERK)1/2 contributes to cytokine-induced apoptosis in purified rat pancreatic beta-cells. *Eur Cytokine Netw* 11:267-274, 2000
200. Khoo S, Cobb MH: Activation of mitogen-activating protein kinase by glucose is not required for insulin secretion. *Proc Natl Acad Sci U S A* 94:5599-5604, 1997
201. Benes C, Poitout V, Marie JC, Martin-Perez J, Roisin MP, Fagard R: Mode of regulation of the extracellular signal-regulated kinases in the pancreatic beta-cell line MIN6 and their implication in the regulation of insulin gene transcription. *Biochem J* 340 (Pt 1):219-225, 1999
202. Frodin M, Sekine N, Roche E, Filloux C, Prentki M, Wollheim CB, Van Obberghen E: Glucose, other secretagogues, and nerve growth factor stimulate mitogen-activated protein kinase in the insulin-secreting beta-cell line, INS-1. *J Biol Chem* 270:7882-7889, 1995
203. Arnette D, Gibson TB, Lawrence MC, January B, Khoo S, McGlynn K, Vanderbilt CA, Cobb MH: Regulation of ERK1 and ERK2 by glucose and peptide hormones in pancreatic beta cells. *J Biol Chem* 278:32517-32525, 2003
204. Buteau J, Foisy S, Rhodes CJ, Carpenter L, Biden TJ, Prentki M: Protein kinase Czeta activation mediates glucagon-like peptide-1-induced pancreatic beta-cell proliferation. *Diabetes* 50:2237-2243, 2001
205. Benes C, Roisin MP, Van Tan H, Creuzet C, Miyazaki J, Fagard R: Rapid activation and nuclear translocation of mitogen-activated protein kinases in response to physiological concentration of glucose in the MIN6 pancreatic beta cell line. *J Biol Chem* 273:15507-15513, 1998
206. Khoo S, Griffen SC, Xia Y, Baer RJ, German MS, Cobb MH: Regulation of insulin gene transcription by ERK1 and ERK2 in pancreatic beta cells. *J Biol Chem* 278:32969-32977, 2003
207. Longuet C, Broca C, Costes S, Hani el H, Bataille D, Dalle S: Extracellularly regulated kinases 1/2 (p44/42 mitogen-activated protein kinases) phosphorylate synapsin I and regulate insulin secretion in the MIN6 beta-cell line and islets of Langerhans. *Endocrinology* 146:643-654, 2005
208. Maedler K, Storling J, Sturis J, Zuellig RA, Spinas GA, Arkhammar PO, Mandrup-Poulsen T, Donath MY: Glucose- and interleukin-1beta-induced beta-cell apoptosis requires Ca2+ influx and extracellular signal-regulated kinase (ERK) 1/2 activation and is prevented by a sulfonylurea receptor 1/inwardly rectifying K+ channel 6.2 (SUR/Kir6.2) selective potassium channel opener in human islets. *Diabetes* 53:1706-1713, 2004
209. Paraskevas S, Aikin R, Maysinger D, Lakey JR, Cavanagh TJ, Hering B, Wang R, Rosenberg L: Activation and expression of ERK, JNK, and p38 MAP-kinases in isolated islets of Langerhans: implications for cultured islet survival. *FEBS Lett* 455:203-208, 1999
210. Matter ML, Ruoslahti E: A signaling pathway from the alpha5beta1 and alpha(v)beta3 integrins that elevates bcl-2 transcription. *J Biol Chem* 276:27757-27763, 2001
211. McGilvray ID, Lu Z, Bitar R, Dackiw AP, Davreux CJ, Rotstein OD: VLA-4 integrin cross-linking on human monocytic THP-1 cells induces tissue factor expression by a mechanism involving mitogen-activated protein kinase. *J Biol Chem* 272:10287-10294, 1997
212. Costes S, Longuet C, Broca C, Faruque O, Hani el H, Bataille D, Dalle S: Cooperative effects between protein kinase A and p44/p42 mitogen-activated protein kinase to promote cAMP-responsive element binding protein activation after beta cell stimulation by glucose and its alteration due to glucotoxicity. *Ann N Y Acad Sci* 1030:230-242, 2004
213. Jhala US, Canettieri G, Screaton RA, Kulkarni RN, Krajewski S, Reed J, Walker J, Lin X, White M, Montminy M: cAMP promotes pancreatic beta-cell survival via CREB-mediated induction of IRS2. *Genes Dev* 17:1575-1580, 2003
214. Sen R, Baltimore D: Inducibility of kappa immunoglobulin enhancer-binding protein Nf-kappa B by a posttranslational mechanism. *Cell* 47:921-928, 1986
215. Hayden MS, Ghosh S: Signaling to NF-kappaB. *Genes Dev* 18:2195-2224, 2004

216. Bonizzi G, Karin M: The two NF-kappaB activation pathways and their role in innate and adaptive immunity. *Trends Immunol* 25:280-288, 2004
217. Yamamoto Y, Gaynor RB: Role of the NF-kappaB pathway in the pathogenesis of human disease states. *Curr Mol Med* 1:287-296, 2001
218. Shishodia S, Aggarwal BB: Nuclear factor-kappaB: a friend or a foe in cancer? *Biochem Pharmacol* 68:1071-1080, 2004
219. Chen F: Is NF-kappaB a culprit in type 2 diabetes? *Biochem Biophys Res Commun* 332:1-3, 2005
220. Pomerantz JL, Baltimore D: Two pathways to NF-kappaB. *Mol Cell* 10:693-695., 2002
221. Malek S, Huxford T, Ghosh G: Ikappa Balpha functions through direct contacts with the nuclear localization signals and the DNA binding sequences of NF-kappaB. *J Biol Chem* 273:25427-25435, 1998
222. Palombella VJ, Rando OJ, Goldberg AL, Maniatis T: The ubiquitin-proteasome pathway is required for processing the NF-kappa B1 precursor protein and the activation of NF-kappa B. *Cell* 78:773-785, 1994
223. Schmitz ML, Mattioli I, Buss H, Kracht M: NF-kappaB: a multifaceted transcription factor regulated at several levels. *Chembiochem* 5:1348-1358, 2004
224. Kucharczak J, Simmons MJ, Fan Y, Gelinas C: To be, or not to be: NF-kappaB is the answer--role of Rel/NF-kappaB in the regulation of apoptosis. *Oncogene* 22:8961-8982, 2003
225. Vermeulen L, De Wilde G, Notebaert S, Vanden Berghe W, Haegeman G: Regulation of the transcriptional activity of the nuclear factor-kappaB p65 subunit. *Biochem Pharmacol* 64:963-970, 2002
226. Chen LF, Greene WC: Shaping the nuclear action of NF-kappaB. *Nat Rev Mol Cell Biol* 5:392-401, 2004
227. Campbell KJ, Perkins ND: Post-translational modification of RelA(p65) NF-kappaB. *Biochem Soc Trans* 32:1087-1089, 2004
228. Brown K, Park S, Kanno T, Franzoso G, Siebenlist U: Mutual regulation of the transcriptional activator NF-kappa B and its inhibitor, I kappa B-alpha. *Proc Natl Acad Sci U S A* 90:2532-2536, 1993
229. Scott ML, Fujita T, Liou HC, Nolan GP, Baltimore D: The p65 subunit of NF-kappa B regulates I kappa B by two distinct mechanisms. *Genes Dev* 7:1266-1276, 1993
230. Hoffmann A, Levchenko A, Scott ML, Baltimore D: The IkappaB-NF-kappaB signaling module: temporal control and selective gene activation. *Science* 298:1241-1245, 2002
231. Ting AY, Endy D: Signal transduction. Decoding NF-kappaB signaling. *Science* 298:1189-1190, 2002
232. Arenzana-Seisdedos F, Thompson J, Rodriguez MS, Bachelerie F, Thomas D, Hay RT: Inducible nuclear expression of newly synthesized I kappa B alpha negatively regulates DNA-binding and transcriptional activities of NF-kappa B. *Mol Cell Biol* 15:2689-2696, 1995
233. Guo W, Giancotti FG: Integrin signalling during tumour progression. *Nat Rev Mol Cell Biol* 5:816-826, 2004
234. Karin M, Cao Y, Greten FR, Li ZW: NF-kappaB in cancer: from innocent bystander to major culprit. *Nat Rev Cancer* 2:301-310, 2002
235. Sole C, Dolcet X, Segura MF, Gutierrez H, Diaz-Meco MT, Gozzelino R, Sanchis D, Bayascas JR, Gallego C, Moscat J, Davies AM, Comella JX: The death receptor antagonist FAIM promotes neurite outgrowth by a mechanism that depends on ERK and NF-kapp B signaling. *J Cell Biol* 167:479-492, 2004
236. Yebra M, Filardo EJ, Bayna EM, Kawahara E, Becker JC, Cheresh DA: Induction of carcinoma cell migration on vitronectin by NF-kappa B-dependent gene expression. *Mol Biol Cell* 6:841-850, 1995
237. Are AF, Galkin VE, Pospelova TV, Pinaev GP: The p65/RelA subunit of NF-kappaB interacts with actin-containing structures. *Exp Cell Res* 256:533-544, 2000
238. Barkett M, Gilmore TD: Control of apoptosis by Rel/NF-kappaB transcription factors. *Oncogene* 18:6910-6924, 1999
239. Beg AA, Sha WC, Bronson RT, Ghosh S, Baltimore D: Embryonic lethality and liver degeneration in mice lacking the RelA component of NF-kappa B. *Nature* 376:167-170, 1995
240. Beg AA, Baltimore D: An essential role for NF-kappaB in preventing TNF-alpha-induced cell death. *Science* 274:782-784, 1996
241. Van Antwerp DJ, Martin SJ, Kafri T, Green DR, Verma IM: Suppression of TNF-alpha-induced apoptosis by NF-kappaB. *Science* 274:787-789, 1996

242. Wang CY, Mayo MW, Baldwin AS, Jr.: TNF- and cancer therapy-induced apoptosis: potentiation by inhibition of NF-kappaB. *Science* 274:784-787, 1996
243. Liu ZG, Hsu H, Goeddel DV, Karin M: Dissection of TNF receptor 1 effector functions: JNK activation is not linked to apoptosis while NF-kappaB activation prevents cell death. *Cell* 87:565-576, 1996
244. Bentires-Alj M, Dejardin E, Viatour P, Van Lint C, Froesch B, Reed JC, Merville MP, Bours V: Inhibition of the NF-kappa B transcription factor increases Bax expression in cancer cell lines. *Oncogene* 20:2805-2813, 2001
245. Nozaki S, Sledge Jr GW, Nakshatri H: Repression of GADD153/CHOP by NF-kappaB: a possible cellular defense against endoplasmic reticulum stress-induced cell death. *Oncogene* 20:2178-2185, 2001
246. Romashkova JA, Makarov SS: NF-kappaB is a target of AKT in anti-apoptotic PDGF signalling. *Nature* 401:86-90, 1999
247. Kasibhatla S, Genestier L, Green DR: Regulation of fas-ligand expression during activation-induced cell death in T lymphocytes via nuclear factor kappaB. *J Biol Chem* 274:987-992, 1999
248. Matsui K, Fine A, Zhu B, Marshak-Rothstein A, Ju ST: Identification of two NF-kappa B sites in mouse CD95 ligand (Fas ligand) promoter: functional analysis in T cell hybridoma. *J Immunol* 161:3469-3473, 1998
249. Campbell KJ, Rocha S, Perkins ND: Active repression of antiapoptotic gene expression by RelA(p65) NF-kappa B. *Mol Cell* 13:853-865, 2004
250. Bissell MJ, Barcellos-Hoff MH: The influence of extracellular matrix on gene expression: is structure the message? *J Cell Sci Suppl* 8:327-343, 1987
251. Lin TH, Rosales C, Mondal K, Bolen JB, Haskill S, Juliano RL: Integrin-mediated tyrosine phosphorylation and cytokine message induction in monocytic cells. A possible signaling role for the Syk tyrosine kinase. *J Biol Chem* 270:16189-16197, 1995
252. Qwarnstrom EE, Ostberg CO, Turk GL, Richardson CA, Bomsztyk K: Fibronectin attachment activates the NF-kappa B p50/p65 heterodimer in fibroblasts and smooth muscle cells. *J Biol Chem* 269:30765-30768, 1994
253. Wang W, Passaniti A: Extracellular matrix inhibits apoptosis and enhances endothelial cell differentiation by a NfkappaB-dependent mechanism. *J Cell Biochem* 73:321-331, 1999
254. Scatena M, Almeida M, Chaisson ML, Fausto N, Nicosia RF, Giachelli CM: NF-kappaB mediates alphavbeta3 integrin-induced endothelial cell survival. *J Cell Biol* 141:1083-1093, 1998
255. de Fougerolles AR, Chi-Rosso G, Bajardi A, Gotwals P, Green CD, Koteliansky VE: Global expression analysis of extracellular matrix-integrin interactions in monocytes. *Immunity* 13:749-758, 2000
256. Sporn SA, Eierman DF, Johnson CE, Morris J, Martin G, Ladner M, Haskill S: Monocyte adherence results in selective induction of novel genes sharing homology with mediators of inflammation and tissue repair. *J Immunol* 144:4434-4441, 1990
257. Haskill S, Johnson C, Eierman D, Becker S, Warren K: Adherence induces selective mRNA expression of monocyte mediators and proto-oncogenes. *J Immunol* 140:1690-1694, 1988
258. Rosales C, Juliano R: Integrin signaling to NF-kappa B in monocytic leukemia cells is blocked by activated oncogenes. *Cancer Res* 56:2302-2305, 1996
259. Fan ST, Mackman N, Cui MZ, Edgington TS: Integrin regulation of an inflammatory effector gene. Direct induction of the tissue factor promoter by engagement of beta 1 or alpha 4 integrin chains. *J Immunol* 154:3266-3274, 1995
260. Klein S, De Fougerolles AR, Blaikie P, Khan L, Pepe A, Green CD, Koteliansky V, Giancotti FG: alpha5beta1 Integrin Activates an NF-kappaB-Dependent Program of Gene Expression Important for Angiogenesis and Inflammation. *Mol Cell Biol* 22:5912-5922., 2002
261. Li X, Stark GR: NFkappaB-dependent signaling pathways. *Exp Hematol* 30:285-296, 2002
262. Reddy SA, Huang JH, Liao WS: Phosphatidylinositol 3-kinase in interleukin 1 signaling. Physical interaction with the interleukin 1 receptor and requirement in NFkappaB and AP-1 activation. *J Biol Chem* 272:29167-29173, 1997
263. Sizemore N, Leung S, Stark GR: Activation of phosphatidylinositol 3-kinase in response to interleukin-1 leads to phosphorylation and activation of the NF-kappaB p65/RelA subunit. *Mol Cell Biol* 19:4798-4805, 1999
264. Kane LP, Shapiro VS, Stokoe D, Weiss A: Induction of NF-kappaB by the Akt/PKB kinase. *Curr Biol* 9:601-604, 1999
265. Ozes ON, Mayo LD, Gustin JA, Pfeffer SR, Pfeffer LM, Donner DB: NF-kappaB activation by tumour necrosis factor requires the Akt serine-threonine kinase. *Nature* 401:82-85, 1999

266. Rauch BH, Weber A, Braun M, Zimmermann N, Schror K: PDGF-induced Akt phosphorylation does not activate NF-kappa B in human vascular smooth muscle cells and fibroblasts. *FEBS Lett* 481:3-7, 2000
267. Madge LA, Pober JS: A phosphatidylinositol 3-kinase/Akt pathway, activated by tumor necrosis factor or interleukin-1, inhibits apoptosis but does not activate NFkappaB in human endothelial cells. *J Biol Chem* 275:15458-15465, 2000
268. Gustin JA, Ozes ON, Akca H, Pincheira R, Mayo LD, Li Q, Guzman JR, Korgaonkar CK, Donner DB: Cell type-specific expression of the IkappaB kinases determines the significance of phosphatidylinositol 3-kinase/Akt signaling to NF-kappa B activation. *J Biol Chem* 279:1615-1620, 2004
269. Zhu Y, Culmsee C, Klumpp S, Krieglstein J: Neuroprotection by transforming growth factor-beta1 involves activation of nuclear factor-kappaB through phosphatidylinositol-3-OH kinase/Akt and mitogen-activated protein kinase-extracellular-signal regulated kinase1,2 signaling pathways. *Neuroscience* 123:897-906, 2004
270. Vanden Berghe W, Plaisance S, Boone E, De Bosscher K, Schmitz ML, Fiers W, Haegeman G: p38 and extracellular signal-regulated kinase mitogen-activated protein kinase pathways are required for nuclear factor-kappaB p65 transactivation mediated by tumor necrosis factor. *J Biol Chem* 273:3285-3290, 1998
271. Chen BC, Lin WW: PKC- and ERK-dependent activation of I kappa B kinase by lipopolysaccharide in macrophages: enhancement by P2Y receptor-mediated CaMK activation. *Br J Pharmacol* 134:1055-1065, 2001
272. Jefferies CA, O'Neill LA: Rac1 regulates interleukin 1-induced nuclear factor kappaB activation in an inhibitory protein kappaBalpha-independent manner by enhancing the ability of the p65 subunit to transactivate gene expression. *J Biol Chem* 275:3114-3120, 2000
273. See V, Rajala NK, Spiller DG, White MR: Calcium-dependent regulation of the cell cycle via a novel MAPK--NF-kappaB pathway in Swiss 3T3 cells. *J Cell Biol* 166:661-672, 2004
274. Jiang B, Xu S, Hou X, Pimentel DR, Brecher P, Cohen RA: Temporal control of NF-kappaB activation by ERK differentially regulates interleukin-1beta-induced gene expression. *J Biol Chem* 279:1323-1329, 2004
275. Kwon G, Corbett JA, Rodi CP, Sullivan P, McDaniel ML: Interleukin-1 beta-induced nitric oxide synthase expression by rat pancreatic beta-cells: evidence for the involvement of nuclear factor kappa B in the signaling mechanism. *Endocrinology* 136:4790-4795, 1995
276. Giannoukakis N, Rudert WA, Trucco M, Robbins PD: Protection of human islets from the effects of interleukin-1beta by adenoviral gene transfer of an Ikappa B repressor. *J Biol Chem* 275:36509-36513, 2000
277. Heimberg H, Heremans Y, Jobin C, Leemans R, Cardozo AK, Darville M, Eizirik DL: Inhibition of cytokine-induced NF-kappaB activation by adenovirus-mediated expression of a NF-kappaB super-repressor prevents beta-cell apoptosis. *Diabetes* 50:2219-2224, 2001
278. Baker MS, Chen X, Cao XC, Kaufman DB: Expression of a dominant negative inhibitor of NF-kappaB protects MIN6 beta-cells from cytokine-induced apoptosis. *J Surg Res* 97:117-122, 2001
279. Rehman KK, Bertera S, Bottino R, Balamurugan AN, Mai JC, Mi Z, Trucco M, Robbins PD: Protection of islets by in situ peptide-mediated transduction of the Ikappa B kinase inhibitor Nemo-binding domain peptide. *J Biol Chem* 278:9862-9868, 2003
280. Chang I, Kim S, Kim JY, Cho N, Kim YH, Kim HS, Lee MK, Kim KW, Lee MS: Nuclear factor kappaB protects pancreatic beta-cells from tumor necrosis factor-alpha-mediated apoptosis. *Diabetes* 52:1169-1175, 2003
281. Cardozo AK, Heimberg H, Heremans Y, Leeman R, Kutlu B, Kruhoffer M, Orntoft T, Eizirik DL: A comprehensive analysis of cytokine-induced and nuclear factor-kappa B-dependent genes in primary rat pancreatic beta-cells. *J Biol Chem* 276:48879-48886, 2001
282. Maedler K, Sergeev P, Ris F, Oberholzer J, Joller-Jemelka HI, Spinas GA, Kaiser N, Halban PA, Donath MY: Glucose-induced beta cell production of IL-1beta contributes to glucotoxicity in human pancreatic islets. *J Clin Invest* 110:851-860, 2002
283. Zeender E, Maedler K, Bosco D, Berney T, Donath MY, Halban PA: Pioglitazone and sodium salicylate protect human beta-cells against apoptosis and impaired function induced by glucose and interleukin-1beta. *J Clin Endocrinol Metab* 89:5059-5066, 2004
284. Kaltschmidt B, Kaltschmidt C, Hofmann TG, Hehner SP, Droge W, Schmitz ML: The pro- or anti-apoptotic function of NF-kappaB is determined by the nature of the apoptotic stimulus. *Eur J Biochem* 267:3828-3835, 2000

285. Lin KI, DiDonato JA, Hoffmann A, Hardwick JM, Ratan RR: Suppression of steady-state, but not stimulus-induced NF-kappaB activity inhibits alphavirus-induced apoptosis. *J Cell Biol* 141:1479-1487, 1998
286. Fan C, Yang J, Engelhardt JF: Temporal pattern of NFkappaB activation influences apoptotic cell fate in a stimuli-dependent fashion. *J Cell Sci* 115:4843-4853., 2002
287. Ling Z, Van de Casteele M, Eizirik DL, Pipeleers DG: Interleukin-1beta-induced alteration in a beta-cell phenotype can reduce cellular sensitivity to conditions that cause necrosis but not to cytokine-induced apoptosis. *Diabetes* 49:340-345, 2000
288. Papaccio G, Graziano A, d'Aquino R, Valiante S, Naro F: A biphasic role of nuclear transcription factor (NF)-kappaB in the islet beta-cell apoptosis induced by interleukin (IL)-1beta. *J Cell Physiol*, 2004
289. Bernal-Mizrachi E, Wen W, Shornick M, Permutt MA: Activation of nuclear factor-kappaB by depolarization and Ca(2+) influx in MIN6 insulinoma cells. *Diabetes* 51 Suppl 3:S484-488, 2002
290. Cottet S, Dupraz P, Hamburger F, Dolci W, Jaquet M, Thorens B: cFLIP protein prevents tumor necrosis factor-alpha-mediated induction of caspase-8-dependent apoptosis in insulin-secreting betaTc-Tet cells. *Diabetes* 51:1805-1814, 2002
291. Norlin S, Ahlgren U, Edlund H: Nuclear Factor-{kappa}B Activity in {beta}-Cells Is Required for Glucose-Stimulated Insulin Secretion. *Diabetes* 54:125-132, 2005
292. Kitsiou PV, Tzinia AK, Stetler-Stevenson WG, Michael AF, Fan WW, Zhou B, Tsilibary EC: Glucose-induced changes in integrins and matrix-related functions in cultured human glomerular epithelial cells. *Am J Physiol Renal Physiol* 284:F671-679, 2003
293. Chen HC, Chen CA, Guh JY, Chang JM, Shin SJ, Lai YH: Altering expression of alpha3beta1 integrin on podocytes of human and rats with diabetes. *Life Sci* 67:2345-2353, 2000
294. Anderson SS, Kim Y, Tsilibary EC: Effects of matrix glycation on mesangial cell adhesion, spreading and proliferation. *Kidney Int* 46:1359-1367, 1994
295. Haitoglou CS, Tsilibary EC, Brownlee M, Charonis AS: Altered cellular interactions between endothelial cells and nonenzymatically glucosylated laminin/type IV collagen. *J Biol Chem* 267:12404-12407, 1992