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Invited Review

Sialic acids: Key determinants for invasion by the Apicomplexa

Nikolas Friedrich [a], Stephen Matthews [b], Dominique Soldati-Favre [a,*]

[a] Department of Microbiology and Molecular Medicine, CMU, University of Geneva, 1 Rue Michel-Servet, CH-1211 Geneva 4, Switzerland
[b] Department of Biological Sciences, Centre for Structural Biology, Imperial College London, South Kensington, London SW7 2AZ, UK

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ABSTRACT

Sialic acids are ubiquitously found on the surface of all vertebrate cells at the extremities of glycan chains and widely exploited by viruses and bacteria to enter host cells. Carbohydrate-bearing receptors are equally important for host cell invasion by the obligate intracellular protozoan parasites of the phylum Apicomplexa. Host cell entry is an active process relying crucially on proteins that engage with receptors on the host cell surface and promote adhesion and internalisation. Assembly into complexes, proteolytic processing and oligomerization are important requirements for the functionality of these adhesins. The combination of adhesive proteins with varying stringency in specificity confers some flexibility to the parasite in face of receptor heterogeneity and immune pressure. Sialic acids are now recognised to critically contribute to selective host cell recognition by various species of the phylum.

1. Introduction to the role of glycans in host cell invasion by the Apicomplexa

In vertebrates, all cells are decorated with a dense and complex array of glycan structures, collectively termed the glycocalyx, comprising glycoproteins and glycolipids. The glycans consist of chains of sugar molecules, which may be branched and often terminate in a sugar unit belonging to the class of sialic acids (Sias). There is a remarkable array of modifications on Sias, exceeding that of any other common monosaccharide. Additional diversity arises from a variety of glycosidic linkages from C2 to the underlying glycans. The glycans fulfil diverse functions in fertilisation, development, neural plasticity and immune-related processes (Varki, 2008). The ubiquitous distribution of glycans and in particular Sias in the bodies of vertebrates makes them attractive targets for pathogens. These pathogens express adhesins and toxins that bind to glycans with various degrees of specificity and are communally named lectins. For a subset of lectins, sialic acid constitutes a critical component for recognition, which can be specific for one particular sialylated glycoconjugate. Recognition may depend on a specific conformation or modification of the sialic acid (such as methylation, acetylation, sulfation and phosphorylation) or on the linkage to the underlying sugar chain and its composition (Varki, 1997). In addition, the protein or lipid to which the glycan is attached can be important for recognition.

Apicomplexan parasites express an arsenal of adhesins including lectins essential for productive host cell invasion (Fig. 1). In the context of specific receptors these interactions are believed to determine the host cell preference for each parasite and each stage in their life cycle (Cerami et al., 1992; Galinski et al., 1992; Orlandi et al., 1992). Whilst some of the adhesins are conserved amongst several species, others appear to be restricted to a few or a single species, which probably reflects the differences in host and tissue tropism as part of their life cycles (Anantharaman et al., 2007; Templeton, 2007). The purpose of this review is to recapitulate, compare and contrast the role of Sias in host cell invasion by the Apicomplexa. The currently characterised apicomplexan ligands that recognise Sias and the nature of their host receptors are discussed here in the context of their potential contribution to host range specificity.

2. Mechanism of invasion and role of adhesins

Invasion of host cells by apicomplexans is an active and complex process that is fundamentally different from the entry mechanisms of bacteria and viruses that exploit host endocytic uptake pathways. Apicomplexan parasites carry their own machinery for active penetration into the host cell (Carruthers and Boothroyd, 2007). Invasion is initiated by attachment that may occur in any

* Corresponding author. Tel.: +41 22 379 5672; fax: +41 22 379 5702.
E-mail address: dominique.soldati-favre@unige.ch (D. Soldati-Favre).

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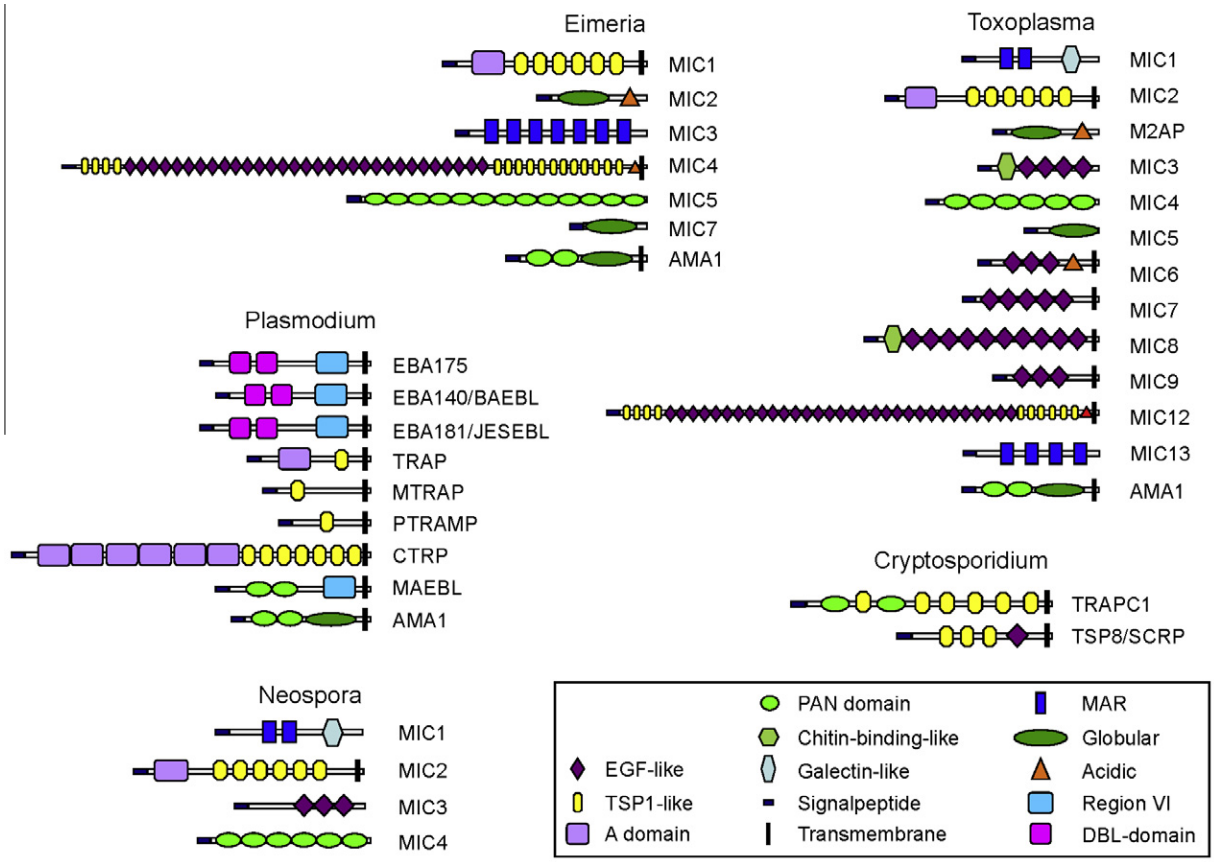

Fig. 1. Representation of a non-exhaustive repertoire of microneme proteins from apicomplexan parasites that exhibit domains involved in protein or carbohydrate interactions. The schematic representation indicates the domain composition of the individual proteins. EGF, epidermal growth factor; TSP, thrombospondin; PAN, plasminogen apple nematode domain; MAR, microneme adhesive repeat. Modified from Tomley and Soldati (2001) and Carruthers and Tomley, 2008.

position of the parasite relative to the host cell and is followed by reorientation such that the parasite apical tip contacts the surface of the host cell. An electron-dense circular structure termed the moving junction is built at the tip, which follows the periphery of the parasite whilst it actively propels itself into the host cell. During this process the host cell plasma membrane progressively invaginates and induces the formation of the parasitophorous vacuole (PV), which is finally sealed behind the parasite. Penetration of the host cell is driven by a parasite actin-myosin motor system located in the space between the parasite plasma membrane and the underlying inner membrane complex (Soldati-Favre, 2008). This motor complex also provides the force for the unique form of substrate-dependent gliding locomotion. Successful host cell invasion relies on the regulated sequential secretion of proteins from two types of membrane-bound organelles, micronemes and rhoptries (Carruthers and Sibley, 1997). Microneme secretion occurs during parasite egress from host cells as well as during gliding locomotion, but mainly upon contact between the parasite and a host cell and is followed by a discharge from the rhoptries that contribute to invasion. Microneme proteins (MICs) are key mediators of cytoadherence, but also play other essential and non-overlapping roles in the invasion process (Carruthers and Tomley, 2008; Soldati-Favre, 2008). Notably, recent studies have highlighted the roles of MICs (TgMIC8 in *Toxoplasma gondii*; PfEBA175 and PfAMA1 in *Plasmodium falciparum*) in the process leading to secretion by the rhoptries (Kessler et al., 2008; Richard et al., 2010; Singh et al., 2010).

The MICs are extensively proteolytically processed during their trafficking along the secretory pathway as well as post-exocytosis. These post-translational events have been studied in detail in *T. gondii* (Dowse and Soldati, 2004). Processing of pro-peptides along the secretory pathway is important for the functionality of some adhesive protein complexes. Only after processing is TgMIC3 able to function as an adhesin through its chitin-binding-like (CBL) domain, but this processing event is dispensable for trafficking (Cerede et al., 2002; El Hajj et al., 2008). Removal of the TgM2AP propeptide is critical for stable assembly and efficient secretion of the TgMIC2–M2AP complex from the micronemes onto the parasite surface (Harper et al., 2006). The cysteine protease cathepsin L (TgCPL) that resides in compartments of the late secretory pathway is a candidate for taking part in proteolytic maturation of some MICs (Larson et al., 2009).

During invasion, the MIC complexes anchored onto the parasite surface are excluded from the forming PV at the level of the moving junction (with the exception of TgAMA1) (Alexander et al., 2005; Howell et al., 2005). For this reason MIC complexes that are in excess or are not part of the moving junction are redistributed towards the posterior end of the parasite during invasion, a phenomenon known as capping, which has been experimentally demonstrated for TgMIC2 and TgMIC3 (Carruthers et al., 1999; Garcia-Reguet et al., 2000). At some point during the entry process, tight interactions formed by the different complexes and host cell receptors have to be disengaged and the MICs removed from the parasite surface. This is effectively achieved by proteolytic shedding, which was first described for TgMIC2. The protease responsible for this activity was termed microneme protein protease 1 (MPP1) and shown to be critical for successful invasion (Carruthers et al., 2000; Brossier et al., 2003). Across the phylum several other MICs as well as resident surface proteins related to adhesive function are shed from the surface either within the transmembrane spanning domain

(Opitz et al., 2002; Zhou et al., 2004; Brossier et al., 2005; Baker et al., 2006) or juxtaposed to the membrane (Howell et al., 2003, 2005). MPP1 is a constitutively active rhomboid-like protease located at the parasite plasma membrane and to date the best candidates are TgROM4 and TgROM5 (Brossier et al., 2005; Dowse et al., 2005). Juxtamembrane cleavage in *P. falciparum* is achieved by a subtilisin-like protease PfSUB2 (Harris et al., 2005).

Additional proteolytic activities causing the trimming of MICs on the parasite surface have been described and termed microneme protein protease 2 and 3 (MPP2 and MPP3) (Carruthers et al., 2000; Zhou et al., 2004). MPP2 activity trims TgMIC2 in several steps from the N-terminus up to its A-domain and clips off a 15 kDa fragment from the TgMIC4 carboxy-terminal end encompassing the sixth apple domain (Brecht et al., 2001). The significance of these processing events remains uncertain as parasites cultivated in vitro in the presence of MPP2 inhibitors do not show any obvious defect. Nevertheless, trimming of TgMIC2 enables its interaction with intercellular adhesion molecule 1 (ICAM-1) and was proposed to be important for parasite transmigration through tissues (Barragan et al., 2005).

3. Importance of sialic acids in host invasion by coccidians

Apicomplexan parasites vary in their capacity to invade different cell types. The *T. gondii* tachyzoite, which is the fast replicating form associated with acute infection in virtually all warm blooded animals, can invade almost any nucleated cell. Interestingly, *T. gondii* shares the capacity to invade a broad range of cells with other tissue-cyst-forming enteroparasites. The group of cyst-forming coccidians includes the genera *Toxoplasma*, *Neospora*, *Hammonida*, *Besnoita*, *Sarcocystis* and *Frenkelia*. Whereas specificity for a certain definitive host appears to be high in these genera, they share the ability to infect a broad range of intermediate hosts (Frenkel and Smith, 2003). Examples for non-cyst-forming coccidians are parasites of the genus *Eimeria*. Each species of this genus infects a very limited number of host species, some appearing to be monoxenous (Vetterling, 1976; Levine and Ivens, 1988; Kvicerova et al., 2007).

Emerging evidence indicates that sialoglycoconjugates constitute major determinants for host cell invasion by the coccidians. An early study on *T. gondii* tachyzoites established that the removal of Sias by neuraminidase treatment of host cells dramatically impaired invasion (Monteiro et al., 1998). The importance of sialic acid was also inferred from experiments using a Chinese hamster ovary cell (CHO) mutant (lec2) with strongly reduced surface-expression of Sias. However the identity of the parasite ligand(s) and the details of the corresponding biologically relevant receptors on the host cell surface remained undetermined. More recently, the atomic structure of TgMIC1 uncovered a novel cell-binding motif called microneme adhesive repeat (MAR), which provides a highly specialised structure for glycan discrimination (Blumenschein et al., 2007). Using carbohydrate microarrays, this adhesin was shown to interact selectively with sialylated oligosaccharides and further structural studies of various complexes between TgMIC1 and sialylated oligosaccharides provided high-resolution insights into the mechanism of recognition (Garnett et al., 2009).

Database mining of the apicomplexan genomes revealed the existence of a family of MAR domain-containing proteins that are restricted to coccidians (Friedrich et al., 2010). Interestingly, the apple domain is specific to coccidians as well (Chen et al., 2008) although a divergent fold belonging to the plasminogen, apple, nematode (PAN) superfamily is present in PfAMA1 and this domain is conserved in AMA-1 homologues from all apicomplexans (Chesne-Seck et al., 2005). In *T. gondii*, the MAR family is composed of TgMIC1, TgMIC13 and two other MAR domain-containing proteins (MCPs).

Similar to TgMIC1, TgMIC13 has the potential to be a key player in Sia-dependent host cell invasion. The carbohydrate-binding specificities of TgMIC13 were determined using binding assays performed on carbohydrate microarrays and compared with MAR regions of TgMIC1 and *Neospora caninum* (Nc)MIC1. The dominant binding activity of all three proteins was to α2–3-linked sialyl probes although the relative binding intensities to specific sialyl probes were different. TgMIC13 bound to 4-*O*-acetylated sialyllactose and α2–9-linked disiayl sequences, which were not recognised by TgMIC1 or NcMIC1. The 4-*O*-acetyl-substituted sialic acid has been described in various tissues in mice, especially in the gut; its presence has been documented in a number of other animal species and in trace amounts in humans (Iwersen et al., 2003; Rinninger et al., 2006). Information concerning the occurrence of 2–9-linked sialic acid in animals is limited. It has been reported as a component on a murine neuroblastoma cell line (Inoue and Inoue, 2003) and a human embryonal carcinoma-cell line (Fukuda et al., 1985).

Analyses of data from carbohydrate microarrays revealed that gangliosides GD1a and GT1b are bound by both TgMIC1 and TgMIC13 (Blumenschein et al., 2007; Friedrich et al., 2010). In order to determine whether gangliosides are able to compete with the authentic host cell receptor(s) for binding of TgMIC1 and TgMIC13, cell-binding assays were performed using gangliosides GD1a and GT1b incorporated into liposomes as competitors. Binding of recombinant TgMIC1 and TgMIC13 to the cells was hardly detectable in the presence of 500 μM GD1a or GT1b (Friedrich et al., unpublished data). Lipid-linked oligosaccharide ligands displayed on liposomes can be much more potent inhibitors than free oligosaccharides due to their multivalent presentation. Therefore the nominal concentrations of the liposome inhibition assays cannot be compared with concentrations of free oligosaccharides. Binding of TgMIC1 and TgMIC13 to gangliosides is of particular interest for parasite biology because *T. gondii* cysts are frequently found in the ganglioside-rich brain tissue. In this context, a survey on ToxoDB (http://toxodb.org/toxo/) revealed that TgMIC13 expression is considerably increased upon induction of tachyzoite to bradyzoite differentiation.

Several glycan-probes in the microarray bound strongly by TgMIC1 and TgMIC13 are based on the 3′sialyl-*N*-acetyllactosamine (3′SiaLacNAc) sequence and host cell binding of TgMIC1 and TgMIC13 was abolished at low mM concentrations of free 3′SiaLacNAc (Friedrich et al., unpublished data). This increased affinity is due to additional contacts to the galactose moiety observed in the crystal structure of TgMIC1 in complex with 3′SiaLacNAc (Garnett et al., 2009), and is likely involved in defining the preference for α2–3 linked sialylated glycoconjugates.

TgMIC13 lacks a membrane-spanning domain, and hence may be part of a complex including an escorter that anchors it to the parasite surface during invasion. TgMIC1 interacts with an apple-domain-containing protein, TgMIC4 (Reiss et al., 2001; Saouros et al., 2005). The apple domain is another potential carbohydrate-binding fold and several apple-domain-containing proteins are present in the *T. gondii* genome. Genetic disruption established the non-essential nature of the TgMIC1–MIC4–MIC6 (TgMIC1–4–6) complex (Reiss et al., 2001), however TgMIC1 has a significant role in invasion and contributes to virulence in the mouse model (Cerede et al., 2005; Blumenschein et al., 2007; Sawmynaden et al., 2008). Parasite attachment to host cells is not significantly impaired in the *mic1ko* strain and therefore cannot explain the invasion defect. Recently, in vitro experiments showed that the transmembrane protein TgMIC6 is able to connect to the motor machinery via interaction with the F-actin binding protein aldolase (Zheng et al., 2009). A tryptophan residue in the extreme C-terminus of the cytoplasmic tail of TgMIC6 is believed to be critical for the interaction with aldolase, however a TgMIC6 mutant carrying

a W/A substitution in this position still functionally complements the invasion phenotype in *mic6ko* (Sheiner et al., unpublished data). This result suggests that interaction of TgMIC6 with aldolase might not be important for the function of the TgMIC1–4–6 complex. Alternatively, rearward translocation of the mutant complex over the parasite surface might be achieved through association with other transmembrane MICs capable of interacting with the glideosome, thus making the interaction of TgMIC6 with aldolase redundant.

No carbohydrate-binding activity or cell-adhesive properties were detected for TgMCP3 and TgMCP4, hence their functions remain a mystery (Friedrich et al., 2010). Epitope-tagging of TgMCP3 and TgMCP4 suggest that these proteins are stored in the dense granules and accumulate in the PV. Additional information on localisation by knock-in facilitated in the Ku-80-ko recipient strain or by the generation of antibodies specific to these proteins would be suitable to confirm these results.

Neospora caninum, the closest known cousin of *T. gondii*, infects a wide variety of mammals and is frequently transmitted by congenital transfer. *Neospora caninum* tachyzoites express homologues of the TgMIC1–4–6 complex. NcMIC1 was originally characterised as a sulphated glycosaminoglycan (GAG) binding protein (Keller et al., 2004). However carbohydrate microarray analyses demonstrated specific binding for NcMIC1 to sialyl-oligosaccharides with the special feature of a strong binding to two sulphated sialyl Le^x-related probes (Friedrich et al., 2010).

Neospora caninum also possesses a homologue of TgMIC13 and hence it corroborates the idea that these proteins fulfil conserved functions, possibly in the capacity of these two cyst-forming parasites to invade a broad range of host cells. In support of this view, expressed sequence tags (ESTs) coding for MCPs are present in another tissue-cyst-forming coccidian, *Sarcocystis neurona*, although limited availability of genomic data precluded the complete annotation of the repertoire of MCPs. In addition, three PAN/apple-domain-containing proteins have been characterised in *Sarcocystis muris* and amongst them the two major micronemal antigens *S. muris* lectin (SmL)-1 and -2. At least one of those localises to the moving junction during host cell invasion (Entzeroth et al., 1992; Klein et al., 2003). The lectin activity of SmL-1 depends on dimerization of the protein and haemagglutination assays indicate binding to N-acetyl-galactosamine (Klein et al., 1998). SmL-2 and another lectin, SmL-3, bind to galactose (Klein et al., 2003) similarly to TgMIC4 (Brecht et al., 2001; Matthews, unpublished data).

Eimeria tenella does not spread through tissues and does not form tissue-cysts but expresses at least one MCP, EtMIC3 (Labbe et al., 2005) and one apple domain-containing protein, EtMIC5 (Brown et al., 2000). The EST database and genomic data indicate that more MCPs and apple domain-containing proteins might be present. EtMIC5 is known to form a multimeric complex with EtMIC4, a transmembrane protein composed of multiple thrombospondin (TSP)-1 and epidermal growth factor (EGF)-like domains (Periz et al., 2007). In this study, coimmunoprecipitation experiments failed to detect an interaction between EtMIC5 and EtMIC3 or any additional protein other than EtMIC4. However, sequence and phylogenetic analyses indicate that *Eimeria* MCPs are divergent from those present in the cyst-forming coccidians and therefore might exhibit different properties (Friedrich et al., 2010).

Although the MAR domain appears highly restricted to coccidian parasites, a recent paper describes a putative protein in *Babesia bovis* strains with similarity to TgMIC1, which may extend the repertoire of the MAR family (Silva et al., 2010). Whilst the sequence exhibits some limited similarity with a central region within the TgMIC1-MAR region, key cysteine residues involved in stabilizing disulphide bonds are not conserved and several new cysteine residues are present. More strikingly, the residues involved in binding sialic acid glycans are absent, suggesting that even if this protein does adopt the MAR fold it is unlikely to recognise sialic acid.

The CBL domain may also contribute to the capacity of *T. gondii* to invade a broad range of cell types. This domain is present in TgMIC3, TgMIC8 as well as in two TgMIC8 paralogues named TgMIC8-2 and TgMIC8-3. BLAST searches (http://eupathdb.org/eupathdb/) identified a full set of homologues in *N. caninum* and indicated the presence of the CBL domain in *E. tenella*. Interestingly, a tryptophan residue critical for the adhesive function of TgMIC3 (Cerede et al., 2005) is conserved amongst all sequences. In contrast, BLAST searches failed to identify a CBL domain in the genomes of *Plasmodium*, *Theileria* or *Cryptosporidium*. Intriguingly, some chitin-binding domains can bind to N-acetylneuraminic acid, therefore it is possible that TgMIC8 and TgMIC3 form another sialyl-specific adhesive complex.

A study performed on *Cryptosporidium parvum* revealed that sialic acid on the surface intestinal epithelial cells triggers parasite encystation (Choudhry et al., 2008). This observation suggests that the parasite possesses a molecular sensor for particular sialoglycoconjugates, but the ligand responsible has yet to be identified.

4. Importance of sialic acids in host invasion by haemosporidia

Haemosporidia spend some of their life cycle in the blood cells of vertebrates and include the malaria parasites of the family Plasmodiidae, numerous bird parasites of the family Haemoproteidae, and the piroplasms and related pathogens of cattle belonging to the family Babesiidae. *Plasmodium* and *Babesia* spp. infect humans and animals with each merozoite invading a red blood cell (RBC) and developing into either a gametocyte (gamete precursors) or a meront (schizonts).

Glycophorins are sialoglycoproteins that play an important role in *Babesia* infections. *Babesia bovis* can invade not only bovine RBCs but also human and other animal RBCs in a Sia-dependent manner (Gaffar et al., 2003; Takabatake et al., 2007a,b). Interestingly, α2–3-linked Sias, but not α2–6-linked ones, are present on the surface of bovine RBCs. Although the α2–3-linked Sia-dependent pathway is needed for efficient invasion of host RBCs by *B. bovis*, there might also be alternative pathways. An OB1 variant of *Babesia rodhaini* uses an alternative invasion pathway that is independent of glycophorin A (GPA) on murine RBCs (Takabatake et al., 2009).

In *P. falciparum*, the situation is fairly complicated, as different strains vary in their dependence on Sia-bearing host cell receptors during invasion into RBCs (Dolan et al., 1994; Thompson et al., 2001; Baum et al., 2003; Triglia et al., 2005; Persson et al., 2008). RBC invasion by parasite lines 3D7 and D10 is to a large extent independent of sialic acid, whereas parasite lines W2-mef and T994 preferentially invade RBCs using sialylated receptors. Based on this phenomenon, two invasion pathways have been described, each one defined by separate ligand-receptor interactions. Two families of parasite ligands have been implicated in providing an explanation for these alternative invasion pathways at the molecular level: The Duffy-binding-like or erythrocyte-binding-protein (DBL-EBP) family and the reticulocyte-binding-like (RBL)-family of proteins (Table 1).

The members of the DBL family contain one or more cysteine-rich regions forming the DBL domain, which was originally identified in *Plasmodium vivax* and *Plasmodium knowlesi* (Fang et al., 1991) and appears to be specific to the genus *Plasmodium* (Aravind et al., 2003; Templeton, 2007). Despite poor sequence conservation across the family, the DBL fold appears to be similar in divergent proteins (Tolia et al., 2005; Singh et al., 2006). Proteins of the DBL family recognise different receptors. Some mediate interactions with sialylated glycoproteins, whilst others recognise specific epitopes on proteins. The family can be divided into two subgroups, the DBL-EBP family functioning in RBC invasion by merozoites

Table 1
Plasmodium falciparum ligands of sialic acid (Sia)-dependent and Sia-independent red blood cell (RBC) invasion.

Name of protein	Localisation	RBC binding	Preferential receptor	Invasion pathway
EBA175	Micronemes (Adams et al., 1992)	Sia-dep. (Orlandi et al., 1992)	Glycophorin A (Orlandi et al., 1992)	Sia-dep. and Sia-indep. (Duraisingh et al., 2003a)
EBA140 (BAEBL)	Micronemes (Thompson et al., 2001; Treeck et al., 2006)	Sia-dep. (Maier et al., 2009)	Promiscuous, Glycophorin C (Mayer et al., 2006; Maier et al., 2009)	Sia-dep. (Maier et al., 2003)
EBA181 (JESEBL)	Micronemes (Treeck et al., 2006)	Sia-dep. (Maier et al., 2009)	Unknown	Sia-dep.
EBL-1	Apical (Mayer et al., 2009)	Sia-dep. (Mayer et al., 2009)	Glycophorin B (Mayer et al., 2009)	Sia-dep.
PfRh1	Apical (Rayner et al., 2001)	Sia-dep. (Triglia et al., 2005)	Unknown	Sia-dep. (Triglia et al., 2005)
PfRh2a	Rhoptries (Duraisingh et al., 2003b)	Not detected	Unknown	Sia-indep. (Desimone et al., 2009)
PfRh2b	Rhoptries (Duraisingh et al., 2003b)	Not detected	Unknown	Sia-indep. (Duraisingh et al., 2003b)
PfRh4	Apical(Stubbs et al., 2005)	Sia-indep. (Gaur et al., 2007)	Unknown	Sia-indep. (Stubbs et al., 2005)
PfRh5	Rhoptries, moving junction (Baum et al., 2009)	Sia-indep. (Baum et al., 2009)	Unknown	Sia-indep.

EBA, erythrocyte-binding antigen; PfRh, *P. falciparum* reticulocyte binding-like homologue; sia, sialic acid; dep., dependent; indep., independent.

(Adams et al., 1992) and the PfEMP-1 family of variant surface antigens expressed at the surface of infected RBCs and involved in immune-evasion and cytoadherence of parasitised RBCs to the endothelium (Scherf et al., 2008).

There is only one protein of the DBL-EBP family in *P. vivax*, the Duffy-binding protein (DBP), which binds to the Duffy blood group antigen (Wertheimer and Barnwell, 1989). RBC invasion by *P. vivax* and *P. knowlesi* critically depends on the Duffy blood group antigen (Miller et al., 1975, 1976) which explains why Duffy-negative or heterozygous human populations in West Africa and Papua New Guinea are resistant to *P. vivax* infections (Kasehagen et al., 2007). PvDBP contains a single copy of the DBL domain and recognition of the host DARC receptor (Duffy antigen receptor for chemokines) does not involve glycans (Singh et al., 2006). More recently *P. vivax* strains were demonstrated to infect Duffy-negative people, suggesting that the parasite has overcome its dependence on the Duffy antigen for establishing human blood-stage infection and disease (Menard et al., 2010). Understanding of the molecular basis for this adaptation awaits further investigation.

In *P. falciparum*, the DBL family comprises four proteins: Erythrocyte-binding antigen 175 (EBA-175), erythrocyte-binding antigen 140 (BAEBL/EBA 140), erythrocyte-binding antigen 181 (JESEBL/EBA 181) and erythrocyte-binding ligand-1 (EBL-1). These type I transmembrane proteins share a similar domain structure comprised of a signal peptide, two DBL domains designated F1 and F2 and together named region II, another cysteine-rich domain called region VI, a membrane-spanning domain and a cytoplasmic tail (Fig. 1). All four proteins are stored in the micronemes (Thompson et al., 2001; Treeck et al., 2006; Mayer et al., 2009). The gene of a fifth member, erythrocyte-binding antigen 165 (EBA165), is a pseudogene. Binding of PfEBA181 to RBCs depends on sialic acid (Maier et al., 2009), but the identity of the receptor remains unknown. The other three proteins have been implicated in interactions with the glycophorins (Mayer et al., 2009).

PfEBA175 preferentially binds to a cluster of O-linked sialylated oligosaccharide structures on glycophorin A (Orlandi et al., 1992). The protein is expected to dimerize during receptor recognition, forming six glycan binding sites involving both subunits (Tolia et al., 2005). The crystal structure confirmed that all of these sites are able to accommodate the full O-glycan of glycophorin A. The interaction between PfEBA175 and glycophorin A is considered to be of importance for Sia-dependent RBC invasion by *Plasmodium* merozoites. This is based on results from several studies on parasite invasion into glycophorin A-deficient (Ena-) and glycophorin A and B-deficient (MkMk) RBCs (Miller et al., 1977; Pasvol and Jungery, 1983; Hadley et al., 1987). In addition, analysis of the invasion phenotype of a PfEBA175 knockout in the Sia-independent 3D7 strain revealed that the protein also functions in parasites that use a Sia-independent invasion pathway (Duraisingh et al., 2003a). PfEBA175 preferentially binds to blood type O RBCs and thus was proposed to contribute to the maintenance of the ABH(O) blood group polymorphisms in the human population (Cohen et al., 2009). The ABH(O) antigens are non-sialylated glycan sequences mostly presented on band 3 and band 4.5 glycoproteins and none of them interact directly with PfEBA175. However, A and B antigens induce the formation of sialylated glycan clusters on the RBC surface that modulate binding of PfEBA175 and potentially other sialic acid-binding lectins of *Plasmodium* and other haemosporidia (Cohen et al., 2009).

PfEBA140 recognises more than one sialylated receptor on the RBC (Maier et al., 2009). Although the binding characteristics of the protein are promiscuous and binding to both glycophorin A and B has been observed (Maier et al., 2003), functional data indicates that an N-linked glycan on glycophorin C serves as the main receptor binding site (Mayer et al., 2006; Maier et al., 2009). PfEBA140 is known to exist in at least five variants that are defined by polymorphism in four non-contiguous amino acid positions, which are located in region II encompassing the DBL domains. These variants were proposed to have different receptor specificity (Mayer et al., 2006), however a recent study shows that their binding characteristics are rather similar (Maier et al., 2009). Although some evidence supports the notion that polymorphisms in PfEBA140 and PfEBA181 affect their binding affinity, this sequence variability is more likely to be related to immune selection. Interestingly, the parasite line D10 (cultured for decades in laboratories) lacks the PfEBA140 gene, indicating that this protein is not essential for parasite invasion in vitro (Thompson et al., 2001), however it remains unknown whether such gene loss has occurred in field isolates.

Glycophorin B was proposed to be the receptor for PfEBL-1 and this interaction was shown to be dependent on sialic acid moieties on the RBC protein (Mayer et al., 2009). However, in approximately 50% of *Plasmodium* isolates the PfEBL-1 gene was found to contain a frame-shift mutation, indicating that it is evolving towards becoming a pseudogene. Glycophorin B is highly polymorphic, an indication of strong selective pressure. The frequency of individuals with glycophorin B-null erythrocytes is particularly high in Africans and African-Americans. Interestingly, this frequency is highest (59%) amongst individuals of the tribe Efé that belong to the Mbuti ethnic group living in the Ituri forests of the Democratic

Republic of the Congo. This has led to speculations that glycophorin B loss might have been a consequence of developing resistance against *P. falciparum* (Mayer et al., 2009). In turn, *P. falciparum* might have counteracted this deficiency with the expansion of the DBL-EBP family.

A second family of EBPs exists in *Plasmodium*, which was first described in *P. vivax* (Galinski et al., 1992). Due to the selective binding of the *P. vivax* proteins to reticulocytes, they were named reticulocyte binding proteins, PvRBP-1 and PvRBP-2, however their corresponding receptors on the RBC remain unknown. Homologues were subsequently identified in other *Plasmodium* spp. and were designated as members of the RBL family (Rayner et al., 2001; Triglia et al., 2001). A group of at least 14 homologues in *Plasmodium yoelii* is named the Py235 family. Most interestingly, rodent malaria *P. yoelii yoelii* merozoites originating from a single schizont were found to express distinct members of the Py235 protein family (Preiser et al., 2002). Although the functions of the individual proteins have not been described, this might indicate that the parasite generates diversity in invasion specificity to overcome immune pressure and receptor heterogeneity.

Similar to the expansion of DBL proteins in *P. falciparum*, the two PvRBPs have five homologues in *P. falciparum* called normocyte binding proteins (NBPs) or RBL-homologues PfRh1, PfRh2a, PfRh2b, PfRh4 and PfRh5. Another gene coding for PfRh3 exists but appears to be a pseudogene. All *P. falciparum* RBLs are large (220–350 kD) type I transmembrane proteins of undefined domain structure except PfRh5, which is much smaller and lacks a transmembrane domain, suggesting that it might be part of a protein complex. In contrast to members of the DBL-EBP family, at least some *P. falciparum* RBL proteins have been detected in the rhoptries. Immunoelectron microscopy demonstrated localisation of PfRh2a, PfRh2b and PfRh5 to this organelle (Duraisingh et al., 2003a; Baum et al., 2009). PfRh4 colocalizes with these proteins in schizonts but is located more apically in free merozoites as shown by indirect immunofluorescence assays (Stubbs et al., 2005). Apical localisation was also detected for PfRh1 in free merozoites (Rayner et al., 2001). Interestingly, PfRh5 was found to follow the tight junction during erythrocyte invasion (Baum et al., 2009). Whereas PfRh1 plays an important role in Sia-dependent invasion, PfRh2a, PfRh2b, PfRh4 and PfRh5 have been implicated in the Sia-independent pathway. PfRh2a and PfRh2b were not formally shown to bind to RBCs, whereas PfRh4 and PfRh5 do function as adhesins (Gaur et al., 2007; Baum et al., 2009).

The importance of individual interactions between parasite proteins and corresponding receptors for RBC invasion has been the focus of numerous studies, since those have important implications for vaccine development. However, due to the redundancy of invasion pathways, it has been difficult to assess the individual contributions of parasite ligands to this process. First indications that *Plasmodium* is able to change the use of receptors on the RBC surface came from a study where the Dd2 clone (derived from the W2-mef strain, which relies completely on sialic acid for invasion) could be selected for invasion into neuraminidase-treated RBCs (Dolan et al., 1994). Further evidence for a switch-mechanism between Sia-dependent and Sia-independent invasion pathways in *Plasmodium* came from the functional disruption of PfEBA175 in the W2-mef strain (Reed et al., 2000). These mutant strains were able to invade neuraminidase-treated RBCs, whereas the parental strain was not. Microarray analysis of the PfEBA175 knockout strain and of parental W2-mef parasites either selected or not on neuraminidase-treated RBCs revealed that the switch of invasion pathways was associated with an up-regulation of PfRh4 (Stubbs et al., 2005). PfRh4 is exclusively expressed in several Sia-independent parasite lines including parasites that had undergone the switch to this pathway. Disruption of PfRh4 was impossible in these lines, but was achieved in W2-mef. This knockout mutant was unable to switch to the Sia-independent invasion pathway, demonstrating that the switching mechanism relies on PfRh4 (Stubbs et al., 2005). In contrast both PfRh2a and PfRh2b are dispensable for switching of invasion pathways in the W2-mef strain (Desimone et al., 2009). Detailed characterisation of a PfRh2b knockout invasion phenotype led to the conclusion that the protein functions in a Sia-independent pathway and its disruption is compensated by the increased use of other receptor–ligand interactions (Duraisingh et al., 2003b). A function in Sia-independent invasion was also suggested for PfRh2a (Desimone et al., 2009). PfRh1 has been identified as an important player in Sia-dependent invasion (Triglia et al., 2005). In a given strain the relative amount of PfRh1 expression versus Rh2a and Rh2b levels correlates with the use of Sia-dependent or -independent pathways.

The relevance of this remarkable capacity of the parasite to switch between alternative invasion pathways for a natural infection with the malaria parasite remains an important unanswered question (Bei et al., 2007). The occurrence of these pathways in field isolates has been investigated in two studies in India and The Gambia as well as in another study with isolates from different origins (Perkins and Holt, 1988; Okoyeh et al., 1999; Baum et al., 2003). In summary, considerable variation was found to exist amongst the field isolates in their use of Sia-dependent and -independent invasion pathways. Analysis of invasion inhibitory antibodies acquired by malaria patients in Kenya showed that these were directed against ligands of both invasion pathways (Persson et al., 2008). Proteins of the DBL-EBP and RBL families were found to be amongst the major targets. Therefore it was suggested that *Plasmodium* uses the variation in invasion phenotype for immune-evasion during infection in humans.

PfEBA175, as a dominant invasion ligand, provides one of the most striking hypotheses of the impact of sialic acid recognition on host range specificity (Varki and Gagneux, 2009). The two closely related malaria species *P. falciparum* and *Plasmodium reichenowi* exhibit strong host preferences for humans and chimpanzees, respectively (Martin et al., 2005). A major change took place in the human lineage after divergence from our common ancestor with chimpanzees that was caused by the inactivation of the CMAH gene. In consequence humans cannot produce N-glycolylneuraminic acid (Neu5Gc) from N-acetylneuraminic acid (Neu5Ac) whereas great apes produce a mixture of both and predominantly Neu5Gc. A second critical genetic mutation likely occurred in the *P. falciparum* lineage that evolved from *P. reichenowi* (Rich et al., 2009), which converted EBA-175 with a marked preference for Neu5Gc to the PfEBA175 binding selectively to Neu5Ac (Martin et al., 2005).

5. Concluding remarks

For many lectins, oligomerization was demonstrated to be important, as a strategy to enhance the affinity and specificity toward their respective carbohydrate ligands. Structural studies have highlighted the necessity for PfEBA175 to dimerize prior to interaction with the sialoglycoprotein glycophorin A during RBC invasion (Tolia et al., 2005) (Fig. 2A). Although clear electron density was not observed for the whole carbohydrate in the crystal structure of PfEBA175 in the presence of 3′sialyllactose (3′SiaLac), it was postulated that the dimer would recognise multiple glycans on dimeric glycophorin A (Fig. 2A and B). In TgMIC1, two sialic acid binding sites are located in solvent-exposed, shallow grooves presented on one edge of each MAR domain (Fig. 2C and D). The crystal structure of TgMIC1 in the presence of 3′SiaLacNAc reveals that specific contacts are made to both sialic acid and galactose moieties (Fig. 2C and D). Oligomerization has been reported for several microneme proteins, including the major micronemal antigen of

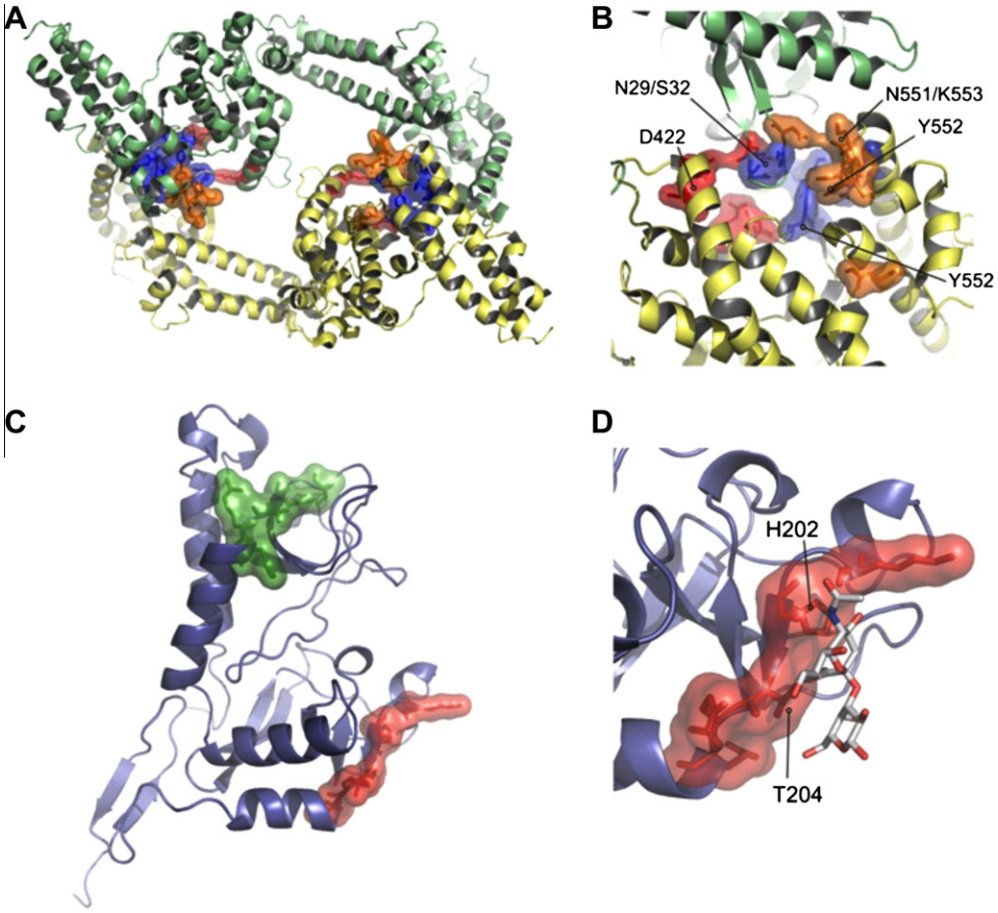

Fig. 2. Comparison of the sialic acid-binding sites for the duplicated DBL domain (together called region II) of erythrocyte binding antigen (EBA)-175 from *Plasmodium falciparum* and the microneme adhesive repeat (MAR) domains of microneme protein 1 from *Toxoplasma gondii* (TgMIC1). (A and B) Crystal structure of the dimeric EBA-175 region II in the presence of 3′SiaLac. EBA-175 region II dimerises in an anti-parallel handshake formation (monomers shown as yellow and green cartoons), which is stabilized by a β-finger from each monomer inserting into an opening of the opposing monomer and creating two deep channels through the dimer. Although clear density was not observed for the whole carbohydrate, three separate binding sites for the sialyl moiety were delineated in each half of the dimer. Key sialic acid interacting residues are shown for each of the three binding sites (red, orange and blue). A total of six sugar-binding sites lie at the dimer interface and deep within the dimeric structure that could accommodate the glycans of a dimeric glycophorin A, the erythrocyte receptor for EBA-175. (C and D) Crystal structure of the tandem MAR domain from TgMIC1 (blue cartoon) in the presence of 3'SiaLacNAc. Good electron density was visualised for the bound 3'SiaLacNAc which, in contrast to the deep channel of EBA-175, interacts with shallow grooves on one edge of the structure. Key side chains from two binding sites are shown in green (MAR1) and red surfaces (MAR2), with the 2,3-sialyllactosamine shown as grey sticks. Although the precise receptor for TgMIC1 is unknown, the arrangement of the binding sites within the crystal structure of TgMIC1 suggests that it may target multi-antennary carbohydrate structures including gangliosides.

S. muris, which is a galactose-specific lectin (Klein et al., 1998) as well as for several lectins in *T. gondii*, the best characterised being TgMIC2 and TgMIC3 (Cerede et al., 2002; Harper et al., 2004). In contrast to PfEBA175, TgMIC1 does not dimerise, but is present in the TgMIC1–4–6 complex, with stoichiometry that allows for the presentation of at least two molecules of TgMIC1 in close proximity. Despite this, the presentation of a single TgMIC1 is functional in invasion, but its arrangement within the complex appears to increase the affinity of the complex to host cell receptor(s) or may allow TgMIC1 to target multi-antennary carbohydrate structures including gangliosides which in turn results in enhanced invasion efficiency (Sawmynaden et al., 2008).

The roles of MICs (TgMIC8, PfEBA175 and PfAMA1) in the process leading to the secretion by the rhoptry organelles (Kessler et al., 2008; Richard et al., 2010; Singh et al., 2010) is an exiting new aspect of receptor–ligand interactions in apicomplexans and of the mechanism of host cell invasion. Molecular genetic studies in *Plasmodium* and *Toxoplasma* suggest that signal transduction is likely mediated by the cytoplasmic domains of these MICs (Gilberger et al., 2003; Treeck et al., 2009). Differences in signalling could provoke a deregulation of parasite organellar exocytosis and could provide a basis to explain switching of invasion pathways in *P. falciparum* since ligands of Sia-dependent and -independent invasion are stored in micronemes and rhoptries, respectively.

Taken together, these findings highlight the necessity to study lectins by applying interdisciplinary approaches. Carbohydrate microarray technology is a powerful tool to screen for binding preferences, which can subsequently be verified with other binding assays in a more physiological context. Structural studies can provide valuable information on the interactions of lectins with their corresponding sugars at the atomic level, revealing critical aspects of recognition which may also be important for drug design. It remains a major challenge to investigate the interactions of lectins in the context of homo- and hetero-oligomer formation and moreover the importance of individual events in the dynamic host-pathogen interplay in vivo. The landscape of glycan structures on the surface of vertebrate cells is constantly evolving and pathogens have to counteract by adapting and expanding their arsenal of lectins accordingly. The implications of this ongoing battle for the evolution of species and in particular the emergence of the human lineage is a fascinating area of research (Varki and Gagneux, 2009).

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