Molecular players of sialic acid-dependent host cell invasion by toxoplasma gondii

FRIEDRICH, Jan Nikolas

Abstract

Toxoplasma gondii causes toxoplasmosis in animals and human and is a model organism for Apicomplexa, a phylum of obligate intracellular parasites including the genus Plasmodium responsible for malaria. Active host cell invasion by these parasites crucially relies on the release of proteins (MICs) from secretory organelles called micronemes onto the parasite surface. MICs engage with receptors on the host cell surface promoting adhesion and internalization. We found that sialic acid, an abundant component of glycoconjugates on all vertebrate cells, is a major determinant for invasion by T. gondii. Two T. gondii MICs, TgMIC1 and TgMIC13, were characterized as the key players of sialic acid-dependent host cell invasion. These two MICs possess domains that adopt a novel fold, termed Microneme Adhesive Repeat (MAR). This domain defines a new protein-family in enteroparasitic coccidians, a subset of apicomplexa including T. gondii. Binding specificities of these proteins might contribute to tissue tropism during infection.

Reference


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Molecular Players Of Sialic Acid-Dependent Host Cell Invasion

By Toxoplasma Gondii

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N.B.- La thèse doit porter la déclaration précédente et remplir les conditions énumérées dans les "Informations relatives aux thèses de doctorat à l'Université de Genève".

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Abstract

Toxoplasma gondii, the causative agent of toxoplasmosis in animals and human, belongs to a diverse phylum of obligate intracellular protozoan parasites, the Apicomplexa which also includes the genus Plasmodium responsible for malaria. Invasion of host cells allows these parasites to scavenge host cell metabolites and provides them with partial protection from the host immune system. Apicomplexan parasites carry a machinery for active host cell invasion which relies on the release of proteins from secretory organelles called micronemes onto the parasite surface. Among the microneme proteins (MICs) characterized to date, a subset forms complexes with non-overlapping functions in host cell recognition and invasion. Adhesive domains in these complexes engage with receptors on the host cell surface and are believed to contribute to host cell attachment and specificity. Apicomplexan parasites vary in their capacity to invade different cell types. P. falciparum blood stage parasites have specialized for invasion into red blood cells of humans and great apes. In contrast, 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 like Neospora caninum and Sarcocystis neurona which are of veterinary importance.

Sialic acid is part of glycoconjugates on the surface of all vertebrate cells and plays a critical role in host cell recognition during bacterial and viral infections. Sialoglycoconjugates are also exploited by the Apicomplexa during host cell invasion. We found that sialic acid is a major determinant for invasion by T. gondii. In this study two T. gondii MICs, TgMIC1 and TgMIC13, were characterized as the key players of sialic acid-dependent host cell invasion. These two MICs possess domains that adopt a novel fold, termed Microneme Adhesive Repeat (MAR). This domain defines a new family of proteins conserved among the group of enteroparasitic coccidians. We speculate that the binding specificities of these proteins contribute to tissue tropism during infection.
Résumé

*Toxoplasma gondii* appartient au phylum des Apicomplexes, qui constitue une large collection de parasites eukaryotiques unicellulaires. Parmi ces parasites, plusieurs sont reconnus comme des pathogènes importants chez les animaux ainsi que chez l’humain. Par exemple, les espèces du genre *Plasmodium* provoquent la malaria et *T. gondii* est responsable de la toxoplasmose, une infection normalement sans conséquence chez les individus immunocompétents mais potentiellement dangereuse chez les personnes dont le système immunitaire est affaibli et pour le foetus. La transmission congénitale peut amener à des complications graves incluant la mort du foetus, des avortements avant terme et des malformations.

Tous les Apicomplexes se propagent obligatoirement à l’intérieur des cellules hôtes ce qui présente plusieurs avantages, notamment une accessibilité aux métabolites de l’hôte et une protection partielle contre la défense immunitaire de l’hôte. Ces parasites possèdent une machinerie complexe qui leur permet d’envahir activement la cellule hôte. Ce processus d’invasion dépend strictement de la sécrétion de protéines par les organelles nommées micronèmes. Il est connu que ces protéines micronemales (MICs) forment des complexes et ont des fonctions non-redondantes durant l’invasion. Ces complexes protéiques reconnaissent et s’attachent à des récepteurs dans la matrice extracellulaire ou à la surface de la cellule hôte. Ainsi ils peuvent potentiellement influencer la spécificité du pathogène pour un certains types de cellule. Les stades érythrocytaires de *P. falciparum* sont spécialisés pour envahir les érythrocytes humains et ceux des primates. Par contre, la forme tachyzoïde du *Toxoplasme* qui se propage rapidement pendant l’infection aigue dans tous les animaux à sang chaud, est capable d’envahir une grande variété de types de cellule. *T. gondii* partage cette capacité d’envahir une variété de types de cellule avec autre parasites entériques comme *Neospora caninum* et *Sarcocystis neurona* qui sont important dans la médecine vétérinaire.

L’acide sialique fait partie des glycoprotéines et des glycolipides et joue un rôle majeur dans la biologie des vertébrés. Comme il est présent à la surface de toutes les cellules des vertébrés, il est exploité par une multitude de bactéries et de virus. Des récepteurs comprenant l’acide sialique sont aussi utilisés par les Apicomplexes pendant l’invasion. Nos résultats montrent que l’acide sialique joue un rôle primordial pour le processus d’invasion chez *T. gondii*. Dans cette étude nous avons caractérisé deux protéines micronemales, TgMIC1 et TgMIC13, qui montrent une reconnaissance spécifique pour l’acide sialique. D’après nos résultats les deux protéines constituent des acteurs clés lors de l’invasion de la
cellule hôte. TgMIC1 et TgMIC13 comprennent un domaine structural nouveau, nommé MAR, qui définit une nouvelle famille de protéines. Les membres de cette famille ont été identifiés uniquement parmi les coccidiens, un group de parasites qui infectent leurs hôtes par la voie entérique. Nous émettons l’hypothèse que la famille de protéines possédant le domaine MAR contribue au tropisme lors de l’infection parasitaire et implique les spécificités d’adhésion individuelles de ces protéines.
Acknowledgements

It’s time to summarize four and a half exciting years that have given me the opportunity to work with and to meet so many nice people. First of all I would like to thank my supervisors Dominique Soldati and Mike Blackman. Dominique, it has been simply great to work with you. I admire your knowledge, enthusiasm and inspiration, all of which make science alive and have continuously stimulated my work. Thanks for your guidance, discussions, and not forgetting your criticism, all from which I have learned so much. Mike, thanks for always being supportive during my time in London, the discussions and your thoughtful advice.

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<th>Description</th>
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<tbody>
<tr>
<td>ABA</td>
<td>Abscisic acid</td>
</tr>
<tr>
<td>AMA</td>
<td>Apical membrane antigen</td>
</tr>
<tr>
<td>APS</td>
<td>Ammonium Persulphate</td>
</tr>
<tr>
<td>BLAST</td>
<td>Basic Local Alignment Search Tool</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>CAT</td>
<td>Chloramphenicol AcetylTransferase</td>
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<tr>
<td>cADPR</td>
<td>cyclic adenosine di-phosphate ribose</td>
</tr>
<tr>
<td>CBL</td>
<td>Chitin-binding-like</td>
</tr>
<tr>
<td>CDPK</td>
<td>Calcium-dependent protein kinase</td>
</tr>
<tr>
<td>CHO</td>
<td>Chinese Hamster Ovary</td>
</tr>
<tr>
<td>CIP</td>
<td>Calf Intestinal alkaline Phosphatase</td>
</tr>
<tr>
<td>CPL</td>
<td>Cathepsin L</td>
</tr>
<tr>
<td>DAPI</td>
<td>Diamidine-2'-phenylindole dihydrochloride</td>
</tr>
<tr>
<td>DBP</td>
<td>Duffy binding protein</td>
</tr>
<tr>
<td>DG</td>
<td>Dense granule</td>
</tr>
<tr>
<td>DHFR-TS or DHFR</td>
<td>DiHydroFolate Reductase-Thymidylate Synthase gene</td>
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<tr>
<td>EBA</td>
<td>Erythrocyte binding antigen</td>
</tr>
<tr>
<td>EBL</td>
<td>Erythrocyte binding ligand</td>
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<tr>
<td>EEF</td>
<td>Exo-erythrocytic forms</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal Growth Factor</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmatic reticulum</td>
</tr>
<tr>
<td>EST</td>
<td>Expressed Sequence Tag</td>
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<tr>
<td>F-actin</td>
<td>Filamentous actin</td>
</tr>
<tr>
<td>FCS</td>
<td>Foetal Calf Serum</td>
</tr>
<tr>
<td>GAP</td>
<td>Gliding Associated Protein</td>
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<tr>
<td>GAG</td>
<td>Glycosaminoglycan</td>
</tr>
<tr>
<td>GFP</td>
<td>Green Fluorescent Protein</td>
</tr>
<tr>
<td>GPI</td>
<td>glycosylphosphatidylinositol</td>
</tr>
<tr>
<td>GRA</td>
<td>Dense granule protein</td>
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<tr>
<td>GST</td>
<td>Glutathione S-transferase</td>
</tr>
<tr>
<td>HA</td>
<td>Hemaglutinin</td>
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<tr>
<td>HPM</td>
<td>Host cell plasma membrane</td>
</tr>
<tr>
<td>HFF</td>
<td>Human Foreskin Fibroblasts</td>
</tr>
<tr>
<td>IFA</td>
<td>Immunofluorescence Assay</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
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<tr>
<td>IMC</td>
<td>Inner membrane complex</td>
</tr>
<tr>
<td>IP3</td>
<td>Inositol-1,4,5-triphosphate</td>
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<tr>
<td>LB</td>
<td>Luria-Bertani</td>
</tr>
<tr>
<td>MAC</td>
<td>Membrane attack complex</td>
</tr>
<tr>
<td>MAR</td>
<td>Microneme Adhesive Repeat</td>
</tr>
<tr>
<td>MARR</td>
<td>MAR-region</td>
</tr>
<tr>
<td>MCP</td>
<td>MAR containing protein</td>
</tr>
<tr>
<td>MIC</td>
<td>Microneme protein</td>
</tr>
<tr>
<td>MLC</td>
<td>Myosin Light Chain</td>
</tr>
<tr>
<td>MPA</td>
<td>mycophenolic acid</td>
</tr>
<tr>
<td>MPP1</td>
<td>Microneme Protein Protease 1</td>
</tr>
<tr>
<td>Acronym</td>
<td>Full Form</td>
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<tr>
<td>---------</td>
<td>-----------</td>
</tr>
<tr>
<td>MPP2</td>
<td>Microneme Protein Protease 2</td>
</tr>
<tr>
<td>MPP3</td>
<td>Microneme Protein Protease 3</td>
</tr>
<tr>
<td>MSP</td>
<td>Merozoite surface protein</td>
</tr>
<tr>
<td>MTIP</td>
<td>Myosin Tail Interacting Protein</td>
</tr>
<tr>
<td>NJ</td>
<td>Neighbour Joining</td>
</tr>
<tr>
<td>ORF</td>
<td>Open Reading Frame</td>
</tr>
<tr>
<td>PAF</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamide Gel Electrophoresis</td>
</tr>
<tr>
<td>PAN</td>
<td>Plasminogen, apple, nematode</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PBS-T</td>
<td>PBS with 0.05% Tween-20</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene Glycol</td>
</tr>
<tr>
<td>PF</td>
<td>Perforin</td>
</tr>
<tr>
<td>PFP</td>
<td>Pore-forming protein</td>
</tr>
<tr>
<td>PI-PLC</td>
<td>Phosphoinositol-phospholipase</td>
</tr>
<tr>
<td>PM</td>
<td>Plasma membrane</td>
</tr>
<tr>
<td>PMCA</td>
<td>Plasma membrane type calcium-ATPase</td>
</tr>
<tr>
<td>PV</td>
<td>Parasitophorous vacuole</td>
</tr>
<tr>
<td>PVM</td>
<td>Parasitophorous vacuole membrane</td>
</tr>
<tr>
<td>RBC</td>
<td>Red blood cell</td>
</tr>
<tr>
<td>RON</td>
<td>Rhoaptry neck protein</td>
</tr>
<tr>
<td>ROP</td>
<td>Rhoaptry protein</td>
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<tr>
<td>RT-PCR</td>
<td>Reverse-Transcriptase PCR</td>
</tr>
<tr>
<td>RyR</td>
<td>Ryanodine receptor</td>
</tr>
<tr>
<td>RBL</td>
<td>Reticulocyte binding-like</td>
</tr>
<tr>
<td>RBP</td>
<td>Reticulocyte binding protein</td>
</tr>
<tr>
<td>Rh</td>
<td>Reticulocyte binding protein homologue</td>
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<tr>
<td>SAG</td>
<td>Surface antigen glycoprotein</td>
</tr>
<tr>
<td>SDM</td>
<td>Site Directed Mutagenesis</td>
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<tr>
<td>SDS</td>
<td>Sodium Dodecyl Sulphate</td>
</tr>
<tr>
<td>SERCA</td>
<td>Sarcoplasmic endoplasmic reticulum calcium-ATPase</td>
</tr>
<tr>
<td>Sia</td>
<td>Sialic acid</td>
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<tr>
<td>SRS</td>
<td>SAG1-related superfamily</td>
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<tr>
<td>SUB</td>
<td>Subtilisin-like protease</td>
</tr>
<tr>
<td>TAE</td>
<td>Tris/Acetate/EDTA</td>
</tr>
<tr>
<td>TAP</td>
<td>Tobacco Acid Pyrophosphatase</td>
</tr>
<tr>
<td>TdT</td>
<td>Terminal Deoxynucleotide Transferase</td>
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<tr>
<td>TE</td>
<td>Tris EDTA</td>
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<tr>
<td>TEMED</td>
<td>N,N,N',N'-Tetramethyl-1,2-diaminomethane</td>
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<tr>
<td>TGN</td>
<td>Trans Golgi Network</td>
</tr>
<tr>
<td>TM</td>
<td>Transmembrane</td>
</tr>
<tr>
<td>TMD</td>
<td>Transmembrane Domain</td>
</tr>
<tr>
<td>TPCK</td>
<td>Tosyl Phenylalanine Chloromethyl Ketone</td>
</tr>
<tr>
<td>TRAP</td>
<td>Thrombospondin-related anonymous protein</td>
</tr>
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<td>YPD</td>
<td>Yeast extract-Peptone-Dextrose</td>
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1. Introduction

1.1 The Phylum Apicomplexa

*Toxoplasma gondii* belongs to the phylum Apicomplexa which comprises a large and diverse group of obligate intracellular protozoan parasites defined by a characteristic ultrastructural element, the apical complex (Figure 1.1) (Roos, 2005). This apical complex determines the cell polarity of these protozoans and comprises electron-dense cytoskeletal structures termed the conoid and the polar ring, and two sets of secretory organelles, the club-shaped rhoptries and the smaller round micronemes located in the apical part (Dubey et al, 1998). The polar ring is connected to microtubules that laterally run along the parasite. The microtubules support a layer of flattened vesicular structures which underlie almost the entirety of the parasite plasma membrane forming the inner membrane complex (IMC). As another particularity, in addition to a single mitochondrion most Apicomplexa (except *Cryptosporidium spp.*.) harbor a plant-like plastid, the apicoplast, which is believed to be the relic of a secondary algal endosymbiont (Vaishnava & Striepen, 2006).

Many apicomplexan parasites are of veterinary or clinical importance such as the *Plasmodium* species that are the etiological agent of malaria and *Cryptosporidium* responsible for opportunistic infections in human. Lifestocks are threatened for example by *Theileria spp.* causing East Coast Fever in cattle and *Eimeria tenella* being responsible for coccidiosis in poultry. *T. gondii* infects all warm-blooded animals and humans. While infection in healthy individuals is usually asymptomatic, the parasite can cause severe disseminated disease in immunocompromised individuals and is a danger for the developing foetus (Montoya & Liesenfeld, 2004). Congenital infection can result in prenatal death, spontaneous abortion and neonatal malformations. Besides being an important pathogen *T. gondii* is an attractive model organism for other members of the phylum as the parasite is comparatively easy to manipulate.
Figure 1.1: Ultrastructure of *T. gondii*.

The left panel shows an electron micrograph of a *T. gondii* tachyzoite growing within a parasitophorous vacuole inside a host cell (courtesy of Dr. Jean-Francois Dubremetz). On the right is a schematic illustrating the structural features of a tachyzoite (courtesy of Dr. Tim Dowse). Colours used in this schematic match with the artificial colouration used in the centre panel to indicate the position of the individual structures. PM: Plasma membrane, ER: Endoplasmatic Reticulum, IMC: Inner membrane complex.

This thesis will focus on *Toxoplasma gondii*, but other coccidians which constitute a group of closely related enteroparasites within the phylum Apicomplexa will also be discussed. Although *Plasmodium* is only more distantly related to *T. gondii*, this parasite will be introduced in some detail because of its importance as a pathogen and because of some unique and remarkable features that are important to keep in mind when considering our findings in *T. gondii*. 
Figure 1.2: Tree of life indicating the position of the phylum Apicomplexa. Courtesy of Dr. Bernardo Foth. PS: photosynthesis.

1.1.1 Life Cycles

Apicomplexan parasites have complex life cycles comprising alternating phases of asexual and sexual development. For many, but not all Apicomplexa asexual and sexual development takes place in distinct hosts called the intermediate host and the definitive host respectively. These parasites are heteroxenous in contrast to monoxenous parasites which develop and are transmitted between a single host species. In course of the life cycle the Apicomplexa undergo several differentiation steps to be optimally adapted to each niche within a given host. All invasive stages are called zoites.

*T. gondii* belongs to the coccidia, a large subgroup of apicomplexan enteroparasites (Figure 1.3). In general tissue cyst-forming coccidia are heteroxenous. 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 more or less broad range of intermediate hosts (Frenkel & 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 (Kvicerova et al, 2007; Levine & Ivens, 1988; Vetterling, 1976). *Eimeria* parasites develop in the host intestine and shedding of oocysts with the feces allows transmission.

![Figure 1.3: Evolutionary relationships between members of the phylum Apicomplexa.](image)

To the right are indicated the pathologies caused by the respective organism.

### 1.1.1.1 Life cycle of *T. gondii*

For sexual development *T. gondii* relies on felines, such as the domestic cat, as definitive host. In contrast virtually all warm-blooded animals including humans can serve as intermediate hosts. Individuals can get infected through ingestion of sporulated oocysts originating from the feces of cats (Montoya & Liesenfeld, 2004). Sporulated oocysts are shed from the intestines of the cat, which are extremely long-lived (Dubey et al, 1998). Once ingested the sporozoite transforms into the rapidly proliferating tachyzoite form that spreads through all tissues and establishes infection. This involves invasion of a large variety of host cells. Asexual replication of tachyzoites within a cell proceeds through a lytic cycle (Figure1.4) (Black & Boothroyd, 2000). Following invasion of a host cell, the zoite multiplies within a vacuole by binary fission (endodyogeny (Striepen et al, 2007)) until the progeny are finally released which are able to infect neighboring cells.
The immune response of the host is then believed to bring about the differentiation of the tachyzoite to the bradyzoite form (Weiss & Kim, 2000). Bradyzoites usually persist for the lifespan of the infected individual inside cysts that are mainly found in muscle and brain tissues. In case of a drop in the immune pressure the semi-dormant encysted bradyzoites can be reactivated and redifferentiate into tachyzoites. Stage conversion frequently happens in immunocompromised individuals. Encysted bradyzoites, and to a much lesser extent tachyzoites, are infectious when ingested, for example in undercooked meat (Montoya & Liesenfeld, 2004). In this way the parasite can be transmitted by carnivorism to its definitive host or infect other intermediate hosts (Figure 1.5 summarizes the T. gondii life cycle). The latter route of transmission between intermediate hosts without passage through the definitive host is a unique feature of T. gondii and N. caninum and is believed to have largely contributed to the success of T. gondii in being one of the most widespread pathogens (Su et al, 2003).
1.1.1.2 *Plasmodium* life cycle

Infectious *Plasmodium* sporozoites are transmitted by a mosquito and deposited into the skin of an intermediate host during a blood-meal. While different species of mosquitoes belonging to the family *Anopheles* are shared by all *Plasmodium* species as definitive hosts, the individual parasite species have adapted to development within different intermediate hosts. Five *Plasmodium* species are known to date to infect humans, which are *P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae* and *P. knowlesi* (Collins & Barnwell, 2009). Figure 1.6 summarizes the *P. falciparum* lifecycle.

After injection into the skin, the sporozoite traverses the epithelium and tries to get to the liver (Amino et al, 2008). Following invasion of a hepatocyte, the parasite multiplies and develops into exo-erythrocytic forms (EEFs) inside its vacuole (Sturm et al, 2009). Finally the parasites differentiate into merozoites and are released into the blood-stream thereby initiating a lytic cycle of asexual replication in red blood cells (RBCs). Merozoites multiply by schizogony proceeding through several characteristic morphological steps called the ring stage, the trophozoite stage and the schizont stage. The cycle of asexual replication in RBCs also leads to the formation of gametocytes, the precursors of male and female gametes that initiate the sexual stages once taken up by a mosquito into its midgut during a blood meal. Male gamete formation is associated with exflagellation. Male and female gametes fuse to
from the ookinete which invades the mosquito midgut epithelium and develops into an oocyst near the basal lamina. The oocyst stage is the only replicative phase of the parasite that does not take place within a host cell. Instead the oocyst is surrounded by an inner layer of parasite origin and an outer layer derived from the basal lamina. Replication leads to the generation of sporozoites which travel via the hemolymph, the fluid that bathes the mosquito cavity, to the salivary glands where they form a reservoir rendering the mosquito infectious for life (Matuschewski, 2006). From the salivary glands, sporozoites are then injected again into an intermediate host closing the life cycle.

Figure 1.6: Schematic of the life cycle of *P. falciparum*. (a) The asexual part of the life-cycle in the intermediate host including liver stage and blood stage development. (b) Sexual development within the definitive host. Taken from (Menard, 2005).

1.2 Host cell invasion

Invasion of host cells by *T. gondii* and other apicomplexan parasites is a complex process (Figure 1.7) that is fundamentally different from the entry mechanisms of bacteria and viruses which exploit host endocytic uptake pathways. Apicomplexan parasites carry their own machinery for active penetration into the host cell (Carruthers & Boothroyd, 2007). Invasion is initiated by initial attachment of the parasite to the host cell. Attachment may occur in any position of the parasite relative to the host cell and is followed by reorientation such that the parasite’s apical tip contacts the surface of the host cell. At this point the attachment becomes
tighter. An electron-dense circular structure termed the moving junction is built which remains at the periphery of the parasite while it actively propels itself into the host cell. During this process the host cell plasma membrane (HPM) progressively invaginates, inducing 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 (PM) and the underlying IMC (Soldati-Favre, 2008). This motor complex provides the force for the unique form of substrate-dependant gliding locomotion. The whole machinery that works in gliding motility including this motor complex has been named the “glideosome”. Successful host cell invasion relies on the regulated sequential secretion of proteins from two types of membrane-bound organelles (Carruthers & Sibley, 1997). Discharge of micronemes occurs first, followed by release of proteins from the rhoptries. Proteins stored in these two sets of organelles have been implicated directly in the invasion process. Finally, an additional set of organelles, the dense granules (DGs), secrete their content into the PV in order to establish the parasite in its new environment within the host cell.

Figure 1.7: Host cell invasion by *T. gondii*.
The schematic on the left represents the various steps of invasion from initial attachment to the host cell until the separation of the parasitophorous vacuole containing the parasite from the host cell plasma membrane. MICs are already present on the parasite surface from the start of the process. Subcellular structures of the parasite are coloured in the cartoon on the bottom left corner. Corresponding colours are used for the name of proteins to indicate the structure they are associated with. Taken from (Carruthers & Boothroyd, 2007). SAG: Surface antigen glycoprotein, MyoA: myosin A, AMA1: Apical membrane antigen 1, ROM: Rhomboid-like protease, MJ: Moving junction. On the right: Electron micrograph of an invading *T.gondii* tachyzoite. Courtesy of Dr. J.-F. Dubremetz.
Microneme proteins (MICs) play essential and non-overlapping roles in the invasion process (Carruthers & Tomley, 2008; Soldati-Favre, 2008). A large repertoire of proteins with adhesive properties has been identified that is delivered onto the parasite surface (Figure 1.8). MICs contain a limited number of adhesive domain types which are arranged in different numbers of copies and in various combinations. For a subset of MICs the formation of complexes has been demonstrated which are already assembled in the endoplasmatic reticulum (ER), travel as an entity through the secretory pathway and are finally released prior to invasion. In *T. gondii*, four complexes composed of transmembrane and soluble MICs and in one case rhoptry neck proteins (RONs) with non-overlapping function in invasion have been described. More complexes are, however, likely to contribute to invasion since additional un-characterized transmembrane microneme proteins (TM-MICs) are encoded in the genome. The specific contribution of each of the four complexes to the invasion process has been uncovered by generating conventional or conditional knockouts of the genes coding for the components of the complexes. TgMIC2 is a TM-MIC that belongs to the thrombospondin-related anonymous protein (TRAP)-family conserved across the phylum Apicomplexa. Members of the TRAP family have attracted much attention due to their central role in gliding motility and host cell invasion (Morahan et al, 2009). TgMIC2 and a soluble partner MIC2-associated-protein, TgM2AP form a multimeric complex (Jewett & Sibley, 2004; Rabenau et al, 2001). This complex grips on receptors present on the extracellular matrix or on the surface of host cells while at the same time being connected to the glideosome via their cytoplasmic tails thus acting as force transducers (Buscaglia et al, 2003; Huynh & Carruthers, 2006; Jewett & Sibley, 2003; Zheng et al, 2009). Parasites depleted for TgMIC2 are markedly deficient in host-cell attachment and gliding motility and are unable to invade host cells (Huynh & Carruthers, 2006). A second complex consists of the TM-MIC TgMIC6, and two soluble MICs, TgMIC1 and TgMIC4. Genetic disruption of any of the three corresponding genes suggests a non-essential role (Reiss et al, 2001), even if the complex has been demonstrated to play an important role for invasion *in vitro* and virulence *in vivo* (Blumenschein et al, 2007; Cerede et al, 2005; Sawmynaden et al, 2008). A third complex is formed by association of the soluble protein TgMIC3 with TgMIC8, a TM-MIC whose genetic disruption interferes with secretion of rhoptry proteins, hence preventing junction formation and completion of invasion (Kessler et al, 2008). Finally, the TM-MIC apical membrane antigen 1 (TgAMA1) anchors a fourth complex to the parasite PM. This complex is unique in that it localizes specifically to the moving junction (Alexander et al, 2005) and
contains the rhoptry proteins TgRON2, TgRON4, TgRON5 and TgRON8 (Alexander et al, 2005; Straub et al, 2009) rather than other MICs, suggesting that TgAMA1 operates downstream of TgMIC8 (Kessler et al, 2008). Parasites depleted in TgAMA1 show normal attachment but are defective in rhoptry secretion, fail to create a junction and are consequently unable to invade a host cell (Mital et al, 2005). Parasite motility is not affected in this mutant. The function, properties, proteolytic processing and trafficking of these four complexes (summarized in table 1.1) will be discussed in detail in the following sections.

Figure 1.8: Representation of a non-exhaustive repertoire of microneme proteins from apicomplexan parasites. The schematic indicates the domain composition of the individual proteins.

1.2.1 The “Glideosome”

The glideosome is the actin-myosin motor machinery located between the parasite plasma membrane and the underlying IMC which is necessary for substrate dependent gliding motility and active host cell penetration in Apicomplexa (Daher & Soldati-Favre, 2009; Soldati-Favre, 2008). The current model of how this motor works is as follows (Figure 1.9). The association of the IMC with subpellicular microtubules provides a rigid support to anchor
the motor. In *T. gondii* two proteins called gliding associated proteins (GAP) 45 and GAP50 were found to be embedded in the IMC (Gaskins et al, 2004) anchoring Myosin A (*TgMyoA*) and the myosin light chain 1 (*TgMLC1*) in a relative fixed position. *TgMyoA* is essential for gliding motility and invasion (Meissner et al, 2002b) and “walks” on actin-filaments (F-actin) to generate forces and movements (Dobrowolski et al, 1997; Dobrowolski & Sibley, 1996). *TgMIC2* and possibly other microneme proteins are translocated by this motor complex over the parasite surface towards the posterior. Rearward translocation of *TgMIC2* depends on the interaction of its cytoplasmic tail with the F-actin-binding protein aldolase, which functions as a molecular bridge between *TgMIC2* and the motor complex (Buscaglia et al, 2003; Huynh & Carruthers, 2006; Jewett & Sibley, 2003; Starnes et al, 2009; Zheng et al, 2009). Attachment of the *TgMIC2*-M2AP complex to a receptor on the extracellular matrix or on the host cell surface will then transduce the force generated by the motor complex and bring about a forward movement of the parasite.

**Figure 1.9: The glideosome in *T. gondii*.** (a) Scanning electron micrograph of a gliding tachyzoite. (b) Transmission electron micrograph of a tachyzoite in the process of host cell invasion. N: Nucleus, M: Mitochondrion, DG: Dense Granule, Ap: Apicoplast, MJ: Moving junction, PVM: Parasitophorous vacuolar membrane. (c) Current model of the glideosome machinery engaging with receptors present on the extracellular matrix or on the host cell. IF: Intermediate filament-like, IMP: Intramembrane particles. Taken from (Carruthers & Boothroyd, 2007).
1.2.2 Induced and constitutive secretion

1.2.2.1 Microneme secretion

Since a large repertoire of proteins essential for motility and host cell invasion is stored in the micronemes, the nature and timely control of their secretion has been under intense investigation. Microneme secretion in *T. gondii* is thought to resemble Ca$^{2+}$-mediated exocytosis in other organisms. A major burst of secretion from the micronemes occurs upon contact between the parasite and the host cell (Carruthers & Sibley, 1997). Furthermore, multiple observations on gliding of extracellular parasites show that parasite motility is sustained by oscillating waves of Ca$^{2+}$ (Huynh & Carruthers, 2006; Lovett & Sibley, 2003; Wetzel et al, 2004). More recently direct evidence was obtained that secretion of microneme content occurs already at the point of egress from a host cell because at least one molecular factor critical for egress, the perforin-like protein 1 (TgPLP1), is released from these organelles (Kafsack et al, 2009). This implies that egressing parasites are already fully prepared to enter a neighboring cell, which is important since the parasite does not survive for long in the extracellular environment.

Experiments in *Plasmodium berghei* (Gantt et al, 2000) and *Cryptosporidium parvum* (Chen et al, 2004) provide evidence that the Ca$^{2+}$-dependent secretion pathway for micronemes is conserved among Apicomplexa. A common mechanism for microneme secretion is further supported by the fact that genes coding for proteins with established or hypothetical function in Ca$^{2+}$-dependent signaling are conserved among all members of the phylum (Nagamune & Sibley, 2006). Moreover conserved factors of motility and invasion are stored in these organelles indicating functional equivalence. Consequently a common mechanism for microneme release would be intuitively assumed.

Microneme secretion can be artificially induced in a variety of Apicomplexa by Ca$^{2+}$-ionophore or ethanol treatment, but not in *E. tenella* sporozoites (Wiersma et al, 2004). Instead in this organism microneme secretion can be stimulated by BSA or FCS in a Ca$^{2+}$-independent manner but the significance of this remains unclear (Bumstead & Tomley, 2000; Wiersma et al, 2004).

Among protozoan parasites, the Ca$^{2+}$-signaling pathway is best understood in *T. gondii* (for a review see (Nagamune et al, 2008)). Interestingly Ca$^{2+}$-signaling in apicomplexan parasites shares similarities with the pathways described in plants and these elements are believed to having been acquired from the apicoplast. The four major aspects of research
associated with Ca\textsuperscript{2+}-signalling in \textit{T. gondii} are: (1) Ca\textsuperscript{2+}-homeostasis in the parasite; (2) pathways for intraparasitic Ca\textsuperscript{2+}-release; (3) molecular players of the Ca\textsuperscript{2+}-response leading to microneme secretion; and (4) identification of stimuli leading to Ca\textsuperscript{2+}-signaling and egress. The importance of Ca\textsuperscript{2+}-signaling for parasite biology are underscored by the fact that mutants that do not respond to Ca\textsuperscript{2+}-ionophore treatment are defective in the establishment of infections \textit{in vivo} (Lavine et al, 2007).

\textbf{1.2.2.2 Ca\textsuperscript{2+}-homeostasis}

Microneme secretion in extracellular \textit{T. gondii} parasites relies on release of Ca\textsuperscript{2+} from intraparasitic stores (Lovett et al, 2002). Intraparasitic stores of calcium are the ER, the mitochondrion, the acidocalcisomes and in case of \textit{Plasmodium} also the food vacuole (Nagamune et al, 2008; Rohrbach et al, 2005). However the rapid availability of free Ca\textsuperscript{2+} from the acidocalcisomes has been questioned since most of it is bound to polyphosphates.

Ca\textsuperscript{2+}-homeostasis in protozoan parasites is achieved by several Ca\textsuperscript{2+}-ATPases and ion exchangers. A comparative genomic analysis indicated conservation of a sacroplasmic endoplasmic reticulum Ca\textsuperscript{2+}-ATPase (SERCA) pump, Golgi-type Ca\textsuperscript{2+}-ATPases and a Ca\textsuperscript{2+}/H\textsuperscript{+}-exchanger among the phylum Apicomplexa (Nagamune & Sibley, 2006). SERCA pumps are known to be important for Ca\textsuperscript{2+}-homeostasis (Nagamune et al, 2007) and in \textit{Plasmodium} this pump (PfATPase6) is one of the proposed targets of the antimalarial drug artemisinin. In contrast plasma membrane type Ca\textsuperscript{2+}-ATPases (PMCA) and voltage-gated Ca\textsuperscript{2+}-channels were only found in \textit{T. gondii} (Nagamune & Sibley, 2006). The contribution to Ca\textsuperscript{2+}-homeostasis by the PMCA TgA1 expressed on acidocalcisomes and the parasite PM was confirmed. The TgA1 deficient strain shows elevated Ca\textsuperscript{2+} resting levels, altered microneme secretion as well as impaired infectivity \textit{in vitro} and \textit{in vivo} (Luo et al, 2005). The level of complexity has been further enhanced since the discovery of a PM-located Na\textsuperscript{+}/H\textsuperscript{+} exchanger (TgNHE1) involved in Ca\textsuperscript{2+}-homeostasis in \textit{T. gondii} (Arrizabalaga et al, 2004), implicating that sodium and proton gradients most likely contribute to the balance. Na\textsuperscript{+}/H\textsuperscript{+}-exchanger are present in other Apicomplexa and they were proposed to act either on the plasma membrane or on acidocalcisomes (Arrizabalaga et al, 2004).
1.2.2.3 Ca$^{2+}$-release pathways

Concerning Ca$^{2+}$-release, pharmacological studies have demonstrated that *T. gondii* responds to agonists and antagonists of inositol-1,4,5-triphosphate-receptor (IP3R) and ryanodine receptor (RyR) channels (Carruthers et al, 1999b; Chini et al, 2005; Lovett et al, 2002). This suggests that the primary source of free Ca$^{2+}$ for intraparasitic signaling is the ER. Release of Ca$^{2+}$ from intraparasitic IP3-sensitive stores has also been monitored in *Plasmodium* (Passos & Garcia, 1998). Similar to the situation in plants (that also respond to IP3R and RyR agonists), both families of intracellular Ca$^{2+}$ release channels appear to be absent from all apicomplexan genomes studied to date suggesting that the responsive channels are of divergent nature (Nagamune & Sibley, 2006). However, a physiological role of a RyR in *T. gondii* is corroborated by the characterization of cyclase and hydrolase activities involved in the turnover of the secondary messenger cyclic ADP ribose (cADPR) (Chini et al, 2005) which is a natural ligand of RyRs. Furthermore Chini *et al.* confirmed the existence of two separate pathways for Ca$^{2+}$-signalling involving IP3Rs and RyRs and leading to microneme secretion. Furthermore, microneme secretion in *T. gondii* can be stimulated with ethanol probably through activation of a phosphoinositol-phospholipase C (PI-PLC), which could generate IP3 (Carruthers et al, 1999b). Of note, all Apicomplexans have a delta type PI-PLC. TgPI-PLC has been characterized and localizes to the cytoplasmic face of the parasite plasma membrane (Fang et al, 2006). However a PI-PLC inhibitor previously shown to cause a block in permeabilization-induced egress (Moudy et al, 2001) was ineffective against the recombinant enzyme.

1.2.2.4 Ca$^{2+}$-signaling

A group of Ca$^{2+}$-dependent protein kinases (CDPKs) are believed to act downstream of Ca$^{2+}$-release together with a range of other Ca$^{2+}$-responsive proteins (calmodulin, calnexin-like and caltractin-like proteins). These CDPKs are otherwise only present in algae and plants and are therefore considered as potential drug targets. Inhibitor studies in *T. gondii* suggest that TgCDPK1 is necessary for microneme secretion and motility (Dobrowolski et al, 1997; Kieschnick et al, 2001). In addition, another class of protein kinases has been implicated in microneme secretion in *T. gondii* and *E. tenella* (Wiersma et al, 2004). Pharmacological
studies suggest that a cyclic GMP-dependant protein kinase (cGMP-PK) is acting downstream of Ca$^{2+}$-dependant events.

Pharmacological studies in *Plasmodium* support a role for PfCDPK1 which is most closely related to TgCDPK3 in motility. PfCDPK1 is believed to be responsible for the phosphorylation of two components of the motor complex that drives parasite motility: The myosin light chain, called myosin tail interacting protein (PfMTIP) and the membrane-anchoring protein PfGAP45 (Green et al, 2008; Kato et al, 2008). For this purpose, PfCDPK1 is suitably located in being anchored in the plasma membrane. A specific inhibitor (purfalcamin) of this enzyme blocks host cell invasion by *T. gondii* tachyzoites (Kato et al, 2008). Furthermore, a compound related to KT5926 (K252a) shown to be active against PfCDPK1 inhibits invasion of *Plasmodium* merozoites into RBCs at low concentrations (Green et al, 2008). Collectively the data suggests that PfCDPK1 is essential for blood stage parasites.

### 1.2.2.5 Rhoptry secretion

Rhoptry secretion appears to rely on contact with a host cell since it has been observed only during invasion following discharge of the micronemes and reorientation of the parasite but before formation of the moving junction (Alexander et al, 2005; Carruthers & Sibley, 1997; Mital et al, 2005). The stimulus and exact molecular mechanism by which rhoptry secretion is elicited remains unknown. From analysis of conditional knockout strains for TgAMA1 and TgMIC8 it was concluded that the two complexes defined by TgMIC8 and TgAMA1 are working upstream of rhoptry secretion (Kessler et al, 2008; Mital et al, 2005). Interestingly, recent work in *P. falciparum* has highlighted an essential role for the cytoplasmic domain of PfAMA1 in a step following reorientation (Treeck et al, 2009). It is therefore conceivable that the PfAMA1 cytoplasmic domain is involved in signal transduction connected to the discharge of the rhoptries.

Proteins are stored in two distinct locations within the club-shaped rhoptries. ROPs (for rhoptry protein) are stored in the bulb, whereas those located in the duct of the rhoptries are called rhoptry neck proteins (RONs). Secretion is believed to take place through the duct and an opening at the apical tip of the parasite releasing the proteins into the host cell cytoplasm either within empty-vacuoles (e-vacuoles) or as soluble proteins (Boothroyd & Dubremetz, 2008). The mechanistic details on how injection is achieved remain a mystery,
but patch-clamp experiments have detected a break in the host plasma membrane very early during the invasion process (Suss-Toby et al, 1996). Release of RONs appears to come first and is followed by the release of the ROPs. After secretion, ROPs have been found in various locations either in the lumen of the nascent PV (TgROP1), associated to the PV membrane (TgROP2 family) or inside the host cell nucleus (TgROP16). In contrast, all released RONs have so far been specifically localized to the moving junction being associated with the membrane of the nascent PV (Besteiro et al, 2009). Whereas TgRON4 and TgRON8 have been located on the cytoplasmic face of the host cell PM, TgRON2 and TgRON5 were proposed to be inserted into this membrane through membrane spanning domains however, as for most putative transmembrane rhoptry proteins, their true topology remains to be established.

1.2.2.6 Dense granule secretion

In *T. gondii* proteins secreted through the dense granules are called granule proteins (GRAs) and are anticipated to fulfill important roles in the remodeling of the PV (Coppens et al, 2006; Mercier et al, 2002). Although a major burst of secretion from the dense granules occurs shortly after host cell invasion (Carruthers & Sibley, 1997), release of proteins in a constitutive, Ca\(^{2+}\)-independent fashion is widely accepted (Chaturvedi et al, 1999). In numerous examples soluble proteins that do not or are prevented to associate with transmembrane proteins containing specific targeting information (escorters) were delivered to the PV via the dense granules (Karsten et al, 1998; Reiss et al, 2001; Striepen et al, 1998; Striepen et al, 2001). Therefore the dense granules are considered to be the default pathway for secretion of soluble proteins in *T. gondii*. The requirements for transmembrane proteins to be secreted through the DGs are not completely understood but seem to be parasite specific (Gendrin et al, 2008). Since all GRA proteins with predicted TMDs are targeted to specific subcompartments of the PV and do not appear to associate with the parasite PM, it remains unclear if the parasite uses these organelles for delivery of endogenous transmembrane and GPI-anchored proteins onto the PM. However, some foreign and mutant TM-proteins were found to be secreted onto the parasite PM via the dense granules (Hoppe et al, 2000; Karsten et al, 1998; Opitz et al, 2002).
1.2.3 Protein trafficking to secretory organelles

Secretory proteins are dispatched from the trans-Golgi-network (TGN) to the different organelles. Post-Golgi sorting in *T. gondii* is believed to resemble in parts the sorting mechanisms of vesicles to secretory organelles in higher eukaryotes. Both tyrosine-based and dileucine motifs in the cytoplasmic tails of type I transmembrane proteins have been identified to mediate targeting to micronemes and rhoptries (Di Cristina et al, 2000; Hoppe et al, 2000; Ngo et al, 2003). In addition an unusual phenylalanine-based motif exposed to the cytosol was suggested to be a targeting determinant for polytopic transmembrane proteins to the micronemes (Sheiner et al, 2008). The tyrosine-based sorting motif might be conserved in *P. falciparum* as mutation of this motif in the cytoplasmic tail of the micronemal protein PfTRAP resulted in its mistargeting (Bhanot et al, 2003). All soluble rhoptry and microneme proteins are believed to travel to the correct organelle after assembly of complexes including transmembrane proteins that carry the appropriate targeting information. These transmembrane proteins have therefore been termed escorters and need to recruit their cargo at an early stage. Indeed, complex formation of the two soluble proteins TgMIC1 and TgMIC4 with the type I transmembrane protein TgMIC6 was shown to take place already in the ER (Reiss et al, 2001). In this case the appropriate loading of the escorter TgMIC6 with its cargo is controlled through TgMIC1 by assisting in the proper folding of TgMIC6 which otherwise cannot leave the ER (Saouros et al, 2005). Similar kind of quality control checkpoints might exist for other complexes. For example, in the absence of soluble TgM2AP (*m2apko*), its escorter TgMIC2 partially accumulates in the early secretory pathway, which in this case leads to a clear defect in host cell entry by the parasite (Harper et al, 2006).

Several studies on microneme targeting support the idea that some proteins and complexes should associate with yet unidentified escorters (Kessler et al, 2008; Treeck et al, 2006; Treeck et al, 2009). In some cases the protein domains that bring about correct targeting and therefore should mediate the association with the escorter have been identified. For example analysis of deletion mutants of *P. falciparum* EBL proteins showed that a conserved cysteine-rich region (region VI) is required for their microneme targeting (Treeck et al, 2006). In case of the GPI-anchored subtilisin-like protease TgSUB1, the prodomain appears to be essential for correct sorting as its fusion to GFP brings the reporter to the micronemes (Binder et al, 2008).
Accumulating data also suggest a role for propeptide sequences present in several MICs in microneme targeting and were proposed to interact with luminal receptors or lipid rafts supporting correct targeting as has been observed in other organisms (Harper et al, 2006). These propeptides are proteolytically removed post-Golgi but before the proteins reach the micronemes. Deletion of the propeptide in TgM2AP resulted in retention of the TgMIC2-M2AP complex in an endosome-like compartment (Harper et al, 2006). Similarly, a TgMIC5 mutant lacking the propeptide failed to traffic to the micronemes (Brydges et al, 2008). Of note, the TgMIC3 propeptide in combination with any of the EGF domains was shown to be the minimum requirement for targeting to the micronemes (El Hajj et al, 2008).

1.2.4 Microneme protein proteolysis

Proteolytic processing has been observed for several microneme proteins during transport to the micronemes and on the parasite surface. Several of these events are believed to be essential for productive host cell invasion; therefore the molecular identification of the proteases involved is of considerable interest. The proteolytic events that involve the four major invasion complexes in *T. gondii* are recapitulated in figure 1.10.

1.2.4.1 Proteolytic maturation in the secretory pathway

In *T. gondii*, several microneme proteins are known to be proteolytically processed along the secretory pathway at the hands of unknown proteases. TgMIC6 looses its most N-terminal EGF-like domain, probably in the trans-Golgi network (Meissner et al, 2002a), but the significance of this event remains unclear. A mutant in which this domain has been deleted shows no defect in targeting, but the possibility remains that it is involved in “loading” the second EGF domain in TgMIC6 with TgMIC1 and TgMIC4 (Reiss et al, 2001; Sawmynaden et al, 2008).

As mentioned above TgMIC5, TgM2AP and TgMIC3 contain pro-peptides with a potential role in trafficking which are proteolytically removed en route to the micronemes. Processing of the pro-peptides in TgM2AP and TgMIC3 was found to be important for the functionality of the corresponding complexes. Only the processed TgMIC3 is able to function as an adhesin through its chitin-binding-like (CBL) domain (Cerede et al, 2002). Additionally,
it was recently shown that 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 and hence is necessary for efficient invasion (Harper et al, 2006).

The cysteine protease cathepsin L (TgCPL) which resides in compartements of the late secretory pathway is a candidate for taking part in proteolytic maturation of secretory proteins (Larson et al, 2009). LHVS (morpholinurea-leucyl-homophenyl-vinyl sulfone phenyl) is a specific inhibitor of this protease that impairs release of proteins from micronemes and consequently parasite attachment and gliding motility (Larson et al, 2009; Teo et al, 2007). This inhibitor should allow the identification of TgCPL substrates in the near future.

Figure 1.10: Proteolytic processing of microneme protein complexes in T. gondii.
Top: Proteolytic maturation taking place within the secretory pathway. Bottom: Proteolytic events taking place after secretion onto the parasite surface involving MPP1 (intramembrane cleavage), MPP2, MPP3 and potentially other protease activities. The cartoon includes a novel transmembrane MIC TgMIC16 currently under investigation in our laboratory (Sheiner et al., in revision). Red: TgMIC2; Light green: TgM2AP; Yellow: TgMIC4; Light blue: TgMIC1; Dark green: TgMIC6; Blue: TgMIC8; Pink: TgMIC3; Grey: TgAMA1; Dark blue, Orange, Violet: TgRONs.

1.2.4.2 Shedding of MIC-complexes post exocytosis

Most single transmembrane proteins on the parasite surface have been shown to be excluded from the forming PV at the level of the moving junction. For this reason MIC complexes that are in excess or are not part of the moving junction are believed to be redistributed towards the posterior end of the parasite during invasion, a phenomenon known as capping, which has been experimentally confirmed at least for TgMIC2 and TgMIC3 (Carruthers et al, 1999a;
Garcia-Reguet et al, 2000). At some point during the penetration process, the tight interactions formed by the different complexes between the parasite and the host cell have to be disengaged and excess adhesive MICs need to be removed. This is effectively achieved by proteolytic shedding of the MIC complexes from the parasite’s surface. Shedding was first observed for TgMIC2 and the responsible so far unidentified protease was termed microneme protein protease 1 (MPP1) (Carruthers et al, 2000b). Furthermore this cleavage event was shown to be critical for successful invasion (Brossier et al, 2003). As for MIC2, many other micronemal transmembrane proteins related to adhesive function including MIC6 are also shed from the surface. Cell-based cleavage assays, as well as studies in the parasite, have demonstrated that shedding takes place within the TMDs of the complex-anchoring TM-MICs TgMIC2, TgMIC6, TgMIC12 and TgAMA1 (Brossier et al, 2005; Howell et al, 2005; Opitz et al, 2002; Urban & Freeman, 2003; Zhou et al, 2004). Mass spectrometric analyses mapped the intramembrane cleavage to a conserved IAGG motif (in case of TgAMA1 an IAGL motif) in TgMIC6 (Opitz et al, 2002), TgMIC2 (Zhou et al, 2004) and TgAMA1 (Howell et al, 2005). MPP1 is anticipated to be 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).

Sequences similar to the MPP1 cleavage motif have been identified in TMDs of MICs in other Apicomplexa and several observations of surface shedding suggest that intramembrane cleavage during invasion is a conserved feature of the phylum (Opitz et al, 2002). Indeed, intramembrane cleavage was demonstrated for PfEBA175 at a site that is conserved across the DBL-EBP family and it was impossible to obtain a parasite line with a mutated cleavage motif (in both 3D7 and W2-mef strains) suggesting that shedding of this protein is a critical event for parasite survival (O'Donnell et al, 2006). Cell-based assays using two P. falciparum rhomboid proteases PfROM1 and PfROM4 and a range of parasite adhesins involved in invasion suggest intramembrane cleavage not only for the DBL-EBP family members but also for the reticulocyte binding-like (RBL) family of proteins and members of the thrombospondin-related anonymous protein (TRAP)-family (Baker et al, 2006). However, in P. falciparum blood stage parasites the subtilisin-like protease PfSUB2 is known to mediate shedding by juxtamembrane cleavage of the GPI-anchored merozoite surface protein 1 (PfMSP1), and of two transmembrane proteins, Plasmodium thrombospondin-related apical merozoite protein (PTRAMP) and PfAMA1 (Green et al, 2006; Harris et al, 2005). Proteolytic shedding of PfAMA1 can also take place within the TMD and the relative importance of these two cleavage events are under investigation.
(Howell et al, 2005). The prime candidate for the rhomboid-like protease responsible for intramembrane shedding in *Plasmodium* is PfROM4, that is among all candidates phylogenetically most closely related to TgROM4 and TgROM5 (Dowse & Soldati, 2005).

### 1.2.4.3 Trimming on the parasite surface

In *T. gondii* several complexes of microneme proteins undergo proteolytic processing after their release onto the parasite surface. At least two distinct proteolytic activities have been noticed and were termed microneme protein protease 2 and 3 (MPP2 and MPP3) (Carruthers et al, 2000b; Zhou et al, 2004). MPP2 activity trims TgMIC2 in several steps from the N-terminus up to its A-domain (Carruthers et al, 2000b) and clips off a 15kDa fragment from the TgMIC4 carboxy-terminal end encompassing the sixth apple domain (Brecht et al, 2001). In addition TgMIC4 is processed at the amino-terminus by an unknown protease. Furthermore proteomic analysis identified TgM2AP and the GPI-anchored subtilisin-like protease TgSUB1 as substrates of MPP2 (Zhou et al, 2004). The significance of most of these processing events remains obscure as parasites cultivated *in vitro* in the presence of MPP2 inhibitors do not show any obvious defect. However, trimming of TgMIC2 enables its interaction with intercellular adhesion molecule 1 (ICAM-1) and was proposed to be important for transmigration through tissues (Barragan et al, 2005). The primary candidate for MPP2 activity is TgSUB1. A knockout strain has been generated and its characterization should clarify this issue (Binder et al, 2008). Interestingly, MPP2-dependent processing is upregulated in a *mic5ko* strain suggesting that TgMIC5 may either act as an inhibitor on MPP2 or somehow protects MPP2 substrates (Brydges et al, 2006).

MPP3 activity is involved in the trimming of TgM2AP (Zhou et al, 2004). Since no inhibitor profile has been established so far it remains unknown whether other proteolytic events correspond to this activity. More work is required to establish whether this activity is of relevance for parasite invasion.
### Table 1.1: Properties of *T. gondii* protein complexes functioning in host cell invasion.

<table>
<thead>
<tr>
<th>Complex and its function</th>
<th>Complex components</th>
<th>Proteolytic processing</th>
<th>Binding specificity</th>
<th>Connection to glideosome</th>
<th>Trafficking</th>
</tr>
</thead>
<tbody>
<tr>
<td>MIC1-4-6 Host cell invasion</td>
<td>MIC1 (soluble)</td>
<td>no</td>
<td>Sialic acid</td>
<td>Relies on escorter MIC6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MIC4 (soluble)</td>
<td>Trimming post secretion</td>
<td>Galactose (S. Matthews, pers. Communication)</td>
<td>Relies on escorter MIC6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MIC6 (TM-MIC)</td>
<td>Maturation in secretory pathway, Shedding during invasion</td>
<td>No binding</td>
<td>yes</td>
<td>Contains trafficking determinant, relies on MIC1</td>
</tr>
<tr>
<td>MIC2-M2AP Motility and host cell attachment</td>
<td>MIC2 (TM-MIC)</td>
<td>Trimming post secretion, Shedding during invasion</td>
<td>Heparin, ICAM-1</td>
<td>yes</td>
<td>Contains trafficking determinant, relies on M2AP</td>
</tr>
<tr>
<td></td>
<td>M2AP (soluble)</td>
<td>Propeptide cleavage, Trimming post secretion</td>
<td>unknown</td>
<td>Relies on escorter MIC2</td>
<td></td>
</tr>
<tr>
<td>MIC3-MIC8 Rhotry secretion</td>
<td>MIC3 (soluble)</td>
<td>Propeptide cleavage</td>
<td>unknown</td>
<td>Relies on unknown escorter</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MIC8 (TM-MIC)</td>
<td>Shedding during invasion</td>
<td>unknown</td>
<td>unknown</td>
<td>Escorted?</td>
</tr>
<tr>
<td>AMA1-RONs Rhotry secretion, Moving junction formation</td>
<td>AMA1 (TM-MIC)</td>
<td>Shedding during invasion</td>
<td>RON2?</td>
<td>unknown</td>
<td>Escorted (Sheiner <em>et al.</em>, in revision)</td>
</tr>
<tr>
<td></td>
<td>RON2 (TM-protein?)</td>
<td></td>
<td></td>
<td>AMA1?</td>
<td></td>
</tr>
<tr>
<td></td>
<td>RON4 (soluble)</td>
<td></td>
<td></td>
<td>RON2/5/8?</td>
<td></td>
</tr>
<tr>
<td></td>
<td>RON5 (TM-protein?)</td>
<td></td>
<td></td>
<td>RON2/4/8?</td>
<td></td>
</tr>
<tr>
<td></td>
<td>RON8 (soluble)</td>
<td></td>
<td></td>
<td>RON2/4/5?</td>
<td></td>
</tr>
</tbody>
</table>

### 1.2.5 The role of glycans in host cell invasion by the Apicomplexa

Glycans are crucial for the biology of many organisms. In vertebrates all cells are decorated with a dense and complex array of glycanstructures collectively termed the glycocalyx comprising glycoproteins and glycolipids. The glycanstructures consist of a chain of sugar molecules which may be branched and often terminate in a sugar unit belonging to the class of sialic acids (Sia). These glycans fullfil diverse functions in fertilization, development, neural plasticity and immune-related processes (Varki, 2008). The ubiquitous distribution of glycans and in particular sialic acids in the body of vertebrates makes them an attractive target.
for pathogens. These pathogens express adhesins and toxins that bind to glycans with more or less specificity.

Proteins that bind to glycans are collectively named lectins. For a subset of lectins sialic acid is the critical component for recognition. Sialic acid binding lectins again can be very 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), the linkage to the underlying sugar chain and the composition of this chain (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. 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, 1992a; Galinski et al, 1992; Martin et al, 2005; Orlandi et al, 1992). While some of the adhesins are conserved among 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).

1.2.5.1 Glycan recognition during host cell invasion by *T. gondii*

Several studies have implicated recognition of cell surface glycans in host cell invasion by *T. gondii*. In earlier work, sulfated proteoglycans were recognized to play a role (Carruthers et al, 2000a; Ortega-Barria & Boothroyd, 1999). Moreover an important role for sialic acid was inferred from a study on invasion using a CHO cell mutant (lec2) with strongly reduced surface-expression of sialic acid (Monteiro et al, 1998). However in these studies the identity of the parasite ligands and the details of the corresponding biologically relevant receptors on the host cell surface remained undetermined. Glycans are now known to be the target of several *T. gondii* proteins comprising parasite resident surface proteins and MICs.
1.2.5.1.1 A lectin family with conserved function throughout Apicomplexa

TgMIC2 is a member of the thrombospondin-related anonymous protein (TRAP)-family of adhesive proteins conserved throughout the phylum of Apicomplexa. TRAP proteins fulfill essential functions in invasion and substrate-dependent gliding motility in these parasites (Morahan et al, 2009) and are characterized by having one or more copies of an integrin-like A-domain and one or more copies of a type I thrombospondin repeat-like (TSR-1) domain. TgMIC2 binds to the surface of host cells (Carruthers et al, 1999a). More specifically, the A-domain of TgMIC2 was reported to bind to heparin, a highly negatively charged glycosaminoglycan (GAG) composed of a repeated variably sulfated disaccharide unit that is ubiquitously present in the extracellular matrix (Harper et al, 2004). This interaction was shown to be dependent on the multimerization of the protein.

Binding to heparin as well as to sulfatides (sulfo-galactosyl-cerebrosides) was also demonstrated for PfTRAP a protein expressed specifically in the sporozoite stage (Muller et al, 1993; Robson et al, 1995). Numerous studies have attempted to address the relative contribution of the A-domain and the TSR-1 domain to the function of PfTRAP. Recombinant PfTRAP A-domain recapitulates the properties of the full-length protein in binding to heparin, sulfatides and hepatocytes (Akhouri et al, 2004; McCormick et al, 1999). In addition, chemical shift mapping experiments demonstrated weak binding of the TSR-1 domain to heparin indicating possible multiple interaction sites with GAGs (Tossavainen et al, 2006). This dual binding function of the protein has been analysed in P. berghei. In this study parasite strains carrying mutations in the PbTRAP A-domain and/or the TSR-1 domain were generated and demonstrated that both structural elements are important for invasion of the mosquito salivary glands, for invasion of hepatocytes and establishment of a liver infection to a different degree (Matuschewski et al, 2002). In contrast to in vitro experiments on heparin binding by TgMIC2 and PfTRAP, this study suggested a function for a well conserved metal-ion-dependent-adhesion-site (MIDAS) within the A-domain. MIDAS dependent binding of the PbTRAP A-domain to the glycoprotein fetuin-A was recently reported (Jethwaney et al, 2005). Therefore it appears that the A-domain might have more than one binding partner on the cell surface as suggested by another study (Akhouri et al, 2004).
1.2.5.1.2 More parasite-glycan interactions

Several microneme proteins are predicted to harbor TSR-1 domains (as the TRAP-family of proteins described above) and apple domains (Anantharaman et al, 2007). Some apicomplexan proteins containing these domains have been demonstrated to carry lectin properties (Keller et al, 2004; Klein et al, 1998; Muller et al, 2001; Tossavainen et al, 2006) and therefore, homologues and other proteins predicted to contain these domains are suspected to be involved in interactions with host cell surface carbohydrates. Apple domains belong to the plasminogen-apple-nematode (PAN) domain superfamily, which have been implicated in both protein-protein and protein-carbohydrate interactions (Collins et al, 2009; Muller et al, 2001). Two divergent copies of this fold are present in the N-terminal part of PfAMA-1 and are involved in complex formation with the RON proteins (Collins et al, 2009). NcMIC4, composed of six apple domains, binds to lactose and to the GAG chondroitin-sulfate A (Keller et al, 2004). Its homologue TgMIC4 was also suspected to bind to carbohydrates (Brecht et al, 2001) and recent results confirm it to be a galactose binding lectin on its own (Prof. Stephen Matthews, personal communication).

TgMIC3 and TgMIC8 form a complex during invasion and both contain an N-terminal chitin-binding-like domain as well as several EGF domains. In addition TgMIC8 comprises a TMD necessary for anchoring the complex into the parasite PM as well as a C-terminal cytoplasmic tail. As the name indicates most chitin-binding-like domains seem to be involved in protein-carbohydrate interactions (Shen & Jacobs-Lorena, 1999; Wright et al, 1991). Indeed, binding activity of TgMIC3 was detected to a range of nucleated cells (Garcia-Reguet et al, 2000), and this activity could be assigned to its chitin-binding domain (Cerede et al, 2002; Cerede et al, 2005). Studies on recombinant TgMIC3 and TgMIC8 showed that the binding activity of the TgMIC3 chitin-binding-like domain depends on dimerization of the protein achieved through its EGF domains (Cerede et al, 2002). Binding could also be detected for TgMIC8 upon artificial dimerization. Carbohydrate binding experiments did not reveal any interaction of TgMIC3 with the classical ligands N-acetyl-glucosamine, chitobiose or chitotriose, suggesting a different specificity (Cerede et al, 2005). This is in agreement with the fact, that the aromatic residues of this domain that are typically involved in the interaction with a carbohydrate molecule (Wright et al, 1991) are found at different positions in TgMIC3 (Cerede et al, 2005). Mutational studies identified two aromatic residues crucial for the binding function of TgMIC3 and the corresponding mutant parasite lines showed impaired virulence in mice underscoring the importance of this interaction (Cerede et al, 2005).
Binding activity of unknown specificity has been previously ascribed to TgMIC1 which forms a complex together with TgMIC4 and TgMIC6. The glycans structures recognized by TgMIC1 and the importance of this interaction for host cell invasion by the parasite is subject of this thesis and will be discussed in detail.

1.2.5.2 Sialic acid-dependent and Sialic acid-independent invasion pathways in *Plasmodium*

Different strains of *P. falciparum* vary in their dependence on sialic acid bearing host cell receptors during invasion into RBCs (Baum et al, 2003; Dolan et al, 1994; Persson et al, 2008; Thompson et al, 2001; Triglia et al, 2005). For example, 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 were described each one defined by multiple ligand-receptor interactions involved. 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 are responsible for the alternative pathways of invasion (Table 1.2).

1.2.5.2.1 Duffy-binding-like (DBL)-family of proteins

The DBL family of proteins all contain one or more cysteine rich regions forming the DBL-domain originally identified in *P. vivax* and *P. knowlesi* (Fang et al, 1991). This domain appears to be specific to the genus *Plasmodium* (Aravind et al, 2003; Templeton, 2007). Despite poor sequence conservation across the family, the fold of the DBL domains appears to be similar in divergent proteins (Singh et al, 2006; Tolia et al, 2005). Proteins of the DBL family recognize receptors of different nature. Whereas some mediate interactions with sialylated oligosaccharides on glycoproteins, others recognize specific epitopes of proteins. The family can be divided into two subgroups, the DBL-EBP family functioning in RBC invasion by merozoites (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 parasitized 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 & Barnwell, 1989). RBC invasion by *P. vivax* and *P. knowlesi* critically depends on the Duffy blood group antigen (Miller et al, 1976; Miller et al, 1975) 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). The interaction between PvDBP containing a single copy of the DBL domain and the Duffy receptor does not appear to involve glycans (Choe et al, 2005; Hans et al, 2005; Singh et al, 2006).

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, a duplicated DBL domain designated F1 and F2 named together region II, another cysteine-rich domain called region VI, a membrane spanning domain and a cytoplasmic tail. All four proteins are stored in the micronemes (Mayer et al, 2009; Sim et al, 1992; Thompson et al, 2001; Treeck et al, 2006). Binding of PfEBA181 to RBCs is dependent 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 which are major sialoglycoproteins on the surface of the erythrocyte.

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 (M⁺⁺⁻⁻) RBCs (Hadley et al, 1987; Miller et al, 1977; Pasvol & Jungery, 1983). Furthermore, antibodies directed against PfEBA175 were found to strongly inhibit RBC invasion (Sim et al, 1990). In addition, analysis of the invasion phenotype of a PfEBA175 knockout in the Sia-independent 3D7 strain revealed that the protein plays as well a role in the Sia-independent invasion pathway (Duraisingh et al, 2003a).

PfEBA140 recognizes more than one sialylated receptor on the RBC (Maier et al, 2009). Although the binding characteristics of the protein are promiscuous and both glycophorin A and B were suggested to serve as receptors (Maier et al, 2003), the preferential
binding site appears to be an N-linked glycan on glycophorin C (Maier et al, 2009; Mayer et al, 2006). PfEBA140 is known to exist in at least five variants that are defined by polymorphism in four non-contiguous amino acids in region II encompassing the DBL domains. These variants were proposed to have different receptor specificity (Mayer et al, 2006; Mayer et al, 2002), 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 (Maier et al, 2009), 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 if such a 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 about 50% of Plasmodium isolates the PfEBL-1 gene was found to contain a frameshift mutation indicating that it is evolving to be 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%) among individuals of the tribe Efé which 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.
Table 1.2: *P. falciparum* ligands of Sia-dependent and Sia-independent RBC invasion.

<table>
<thead>
<tr>
<th>Name of protein</th>
<th>localisation</th>
<th>RBC binding</th>
<th>Preferential receptor on the RBC</th>
<th>Invasion pathway</th>
</tr>
</thead>
<tbody>
<tr>
<td>PfRh1</td>
<td>Apical (Rayner et al, 2001)</td>
<td>Sia-dep. (Triglia et al, 2005)</td>
<td>unknown</td>
<td>Sia-dep. (Triglia et al, 2005)</td>
</tr>
<tr>
<td>PfRh2a</td>
<td>Rhoptries (Duraisingh et al, 2003b)</td>
<td>Not detected</td>
<td>unknown</td>
<td>Sia-indep. (Desimone et al, 2009)</td>
</tr>
<tr>
<td>PfRh2b</td>
<td>Rhoptries (Duraisingh et al, 2003b)</td>
<td>Not detected</td>
<td>unknown</td>
<td>Sia-indep. (Duraisingh et al, 2003b)</td>
</tr>
<tr>
<td>PfRh4</td>
<td>Apical (Stubbs et al, 2005)</td>
<td>Sia-indep. (Gaur et al, 2007)</td>
<td>unknown</td>
<td>Sia-indep. (Stubbs et al, 2005)</td>
</tr>
<tr>
<td>PfRh5</td>
<td>Rhoptries, Moving junction (Baum et al, 2009)</td>
<td>Sia-indep. (Baum et al, 2009)</td>
<td>unknown</td>
<td>Sia-indep.</td>
</tr>
</tbody>
</table>

1.2.5.2.2 Reticulocyte binding like (RBL)-family of proteins

A second family of erythrocyte binding proteins exists in *Plasmodium* which was first described in *P. vivax* (Galinski et al, 1992). Because of the selective binding of the *P. vivax* proteins to reticulocytes, they were named reticulocyte binding protein (PvRBP) -1 and -2 (Galinski et al, 1992). The corresponding receptors on the RBC are unknown. Homologues were subsequently identified in other *Plasmodium* species and were designated as members of the reticulocyte binding-like or RBL-family (Rayner et al, 2001; Triglia et al, 2001). A group of at least 14 homologues in *P. 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, 1999). Although the function of the individual proteins has 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 (220-350kD) 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 localization of PfRh2a, PfRh2b and PfRh5 to this type of organelle (Baum et al, 2009; Duraisingh et al, 2003b). PfRh4 colocalizes with both proteins in schizonts but is located more apically in free merozoites as shown by immunofluorescent assays (Stubbs et al, 2005). Apical localization 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 has been identified as an important player of sialic acid-dependent invasion, PfRh2a, PfRh2b, PfRh4 and PfRh5 have been implicated in the sialic acid-independent pathway as will be explained in the following section. Whereas PfRh2a and PfRh2b were not formally shown to bind to RBCs, PfRh4 and PfRh5 do qualify as adhesins (Baum et al, 2009; Gaur et al, 2007).

**1.2.5.2.3 Switching between invasion pathways**

The importance of individual interactions between parasite proteins and corresponding receptors for RBC invasion has been the focus of numerous studies, since it has important implications for vaccine development. However, because of 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 completely relies on sialic acid for invasion) could be selected for invasion into neuraminidase-treated RBCs (Dolan et al, 1990; 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 selected or not on neuraminidase-treated RBCs revealed that the switch of invasion pathways was associated with upregulation of PfRh4 (Stubbs et al, 2005). In this study PfRh4 was shown to be 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). Further experiments showed that switching between these invasion pathways is reversible and is accompanied by silencing of PfRh4. In contrast both PfRh2a and PfRh2b were shown to be dispensable for switching of invasion pathways in the W2-mef strain (Desimone et al, 2009).

PfRh2a and PfRh2b were found to show variant expression among several P. falciparum isolates examined (Duraisingh et al, 2003b). In agreement with this observation, both genes could be individually disrupted (in W2mef and 3D7) and a parasite line (D10) lacking Rh2b was identified showing that they are not essential for P. falciparum blood stage parasites in vitro (Desimone et al, 2009; Duraisingh et al, 2003b; Triglia et al, 2001). However two studies showed that antibodies directed against the two proteins inhibit RBC invasion, suggesting a role for PfRh2a and PfRh2b in this process (Duraisingh et al, 2003b; Triglia et al, 2001). Detailed characterization of a PfRh2b knockout invasion phenotype led to the conclusion that the protein functions in a Sia-independent pathway and its disruption causes increased use of other receptor-ligand interactions for compensation (Duraisingh et al, 2003b). A function in Sia-independent invasion was also suggested for PfRh2a (Desimone et al, 2009). Co-immunoprecipitation experiments suggested that PfRh2 might interact with PfRh1, a sialic acid-dependent erythrocyte binding protein (Rayner et al, 2001), however experiments have shown that PfRh2b functions independently of PfRh1 in the parasite (Duraisingh et al, 2003b).

PfRh1 has been identified as an important player in sialic-acid dependent invasion (Triglia et al, 2005). Similar to PfRh2a and PfRh2b, expression levels of this protein vary among different strains and this is related to gene duplication events that occurred in several parasite lines. In a given strain the relative amount of PfRh1 expression versus Rh2a and Rh2b expression levels correlates with the use of Sia-dependent or -independent pathways (Triglia et al, 2005). Strains with a high expression of PfRh1 (such as T994) were found to be reliant on the Sia-dependent pathway whereas strains lacking or with reduced levels of PfRh1 (like 3D7) use the Sia-independent pathway for invasion. Therefore disruption of the gene had no effect on invasion of 3D7 parasites but in the T994 strain resulted in a shift to Sia-
independent interactions (Triglia et al, 2005). However, microarray as well as Western blots analysis of the T994 parental and knockout strains failed to detect any upregulation of members of the RBL- and EBL-DBL families at the transcript or protein level.

What is the relevance of this remarkable capacity of the parasite to switch between alternative invasion pathways for a natural infection with the malaria parasite? 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 (Baum et al, 2003; Okoyeh et al, 1999; Perkins & Holt, 1988). In summary, considerable variation was found to exist among 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 among the major targets. Therefore it was suggested that Plasmodium uses the variation in invasion phenotype for immune evasion during infection in humans. So far no alternative invasion pathways with a switching mechanism have been described in other Apicomplexa.

1.3 Hypothesis and Aims of Project

*T. gondii* parasites are able to invade essentially any nucleated cell, but the molecular basis that makes recognition and invasion of such a broad spectrum of cells possible is only beginning to be understood. Two general concepts of receptor-ligand interactions could explain this phenomenon. Either the parasite ligand(s) displays low specificity and therefore is able to bind to a range of host cell receptors, or alternatively the parasite ligand(s) should recognize a receptor that is common to all different cell types. The *T. gondii* MIC1-4-6 complex has been partly characterized before and indicated a non-essential but important role for invasion in vitro and virulence in vivo (Cerede et al, 2005; Reiss et al, 2001). Adhesive properties were ascribed to TgMIC1 (Fourmaux et al, 1996) and TgMIC4 (Brecht et al, 2001), and TgMIC1 was proposed to bind to lactose (Lourenco et al, 2001). We hypothesized that the TgMIC1-4-6 complex functions early in host cell invasion and is able to mediate recognition of a broad spectrum of host cells.
Carbohydrate microarray analyses and structural studies performed on TgMIC1 by our collaborators Prof. Stephen Matthews and Prof. Ten Feizi (both from Imperial College, London) indicated that TgMIC1 binds to sialic acid. In the light of these results the aims of the PhD project were to:

- Functionally characterize the TgMIC1-4-6 complex and in particular TgMIC1.
- Assess the importance of host cell sialylated glycoconjugates for host cell invasion.
- Identify which is/are the important parasite ligand(s) in sialic acid-dependent invasion.
- Characterize any additional parasite ligands involved.
- Identify the relevant host cell receptors.

2. Materials and Methods

This section is intended to give details on the routinely used techniques during this study. For more specific protocols please refer to the Materials and Methods sections of the scientific articles that are part of this thesis.

2.1 Reagents and Suppliers

All chemicals were obtained from Sigma except where stated. Microbiological culture reagents were obtained from BD Biosciences.

2.1.1 Enzymes

All restriction enzymes were from New England Biolabs (NEB).
Calf Intestinal alkaline Phosphatase (CIP; Q-Biogene)
T4 DNA ligase (Roche)
AmpliTaq DNA polymerase (Applied Biosystems)
TaKaRa LA Taq DNA polymerase (TaKaRa)
T4 DNA polymerase (NEB)
Superscript II Reverse Transcriptase (Invitrogen)

2.1.2 Kits

Nucleobond maxi-prep kit for large scale DNA preparation (BD Biosciences); WizardMini-prep kit for small scale DNA preparation (Promega); Easy Pure DNA purification kit (Biozym); pGEM T-easy kit (Promega); TOPO TA-cloning kit (Invitrogen).
All kits were used according to the manufacturer’s instructions.

2.1.3 Antibodies

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Source</th>
<th>Dilution for western blot or immunofluorescence</th>
</tr>
</thead>
<tbody>
<tr>
<td>mouse anti-myc hybridoma GE10</td>
<td>(Invitrogen™)</td>
<td>1:1000</td>
</tr>
<tr>
<td>mouse anti-Ty1 hybridoma BB2</td>
<td>(Bastin et al, 1996)</td>
<td>1:1000</td>
</tr>
<tr>
<td>mouse anti-HA</td>
<td>Covance™</td>
<td>1:1000</td>
</tr>
<tr>
<td>rabbit anti-TgMIC4</td>
<td>(Brecht et al, 2001)</td>
<td>1:1000</td>
</tr>
<tr>
<td>rabbit anti-TgMLC</td>
<td>(Herm-Gotz et al, 2002)</td>
<td>1:1000</td>
</tr>
<tr>
<td>mouse anti-TgSAG1</td>
<td>kindly provided by JF Dubremetz</td>
<td>1:1000</td>
</tr>
<tr>
<td>rabbit anti-TgCatalase</td>
<td>(Ding et al, 2000)</td>
<td>1:1000</td>
</tr>
<tr>
<td>goat anti-mouse, horse radish peroxidise (HRP) conjugated</td>
<td>Molecular Probes™</td>
<td>1:3000</td>
</tr>
<tr>
<td>goat anti-rabbit, HRP conjugated</td>
<td>Molecular Probes™</td>
<td>1:3000</td>
</tr>
<tr>
<td>goat anti-mouse, Alexa 488 conjugated (green)</td>
<td>Molecular Probes™</td>
<td>1:3000</td>
</tr>
<tr>
<td>goat anti-mouse, Alexa 594 conjugated (red)</td>
<td>Molecular Probes™</td>
<td>1:3000</td>
</tr>
<tr>
<td>goat anti-rabbit, Alexa 488 conjugated (green)</td>
<td>Molecular Probes™</td>
<td>1:3000</td>
</tr>
<tr>
<td>goat anti-rabbit, Alexa 594 conjugated (red)</td>
<td>Molecular Probes™</td>
<td>1:3000</td>
</tr>
</tbody>
</table>
2.2 Solutions

2.2.1 Culture Media

**Table 2.2:** Culture media used in this study

<table>
<thead>
<tr>
<th>Culture Media</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>LB (Luria Bertani) liquid media</td>
<td>1% w/v Bacto-Tryptone, 0.5% w/v yeast extract, 1% w/v NaCl</td>
</tr>
<tr>
<td>LB Agar</td>
<td>LB liquid with 1.5 % w/v agar</td>
</tr>
<tr>
<td>YPD (Yeast extract-Peptone-Dextrose) liquid media</td>
<td>1% w/v yeast extract, 2% w/v peptone, 2% w/v dextrose (glucose)</td>
</tr>
<tr>
<td>YPD agar</td>
<td>YPD liquid with 1.5 % w/v agar</td>
</tr>
<tr>
<td>Dulbecco’s Modified Eagle Medium + 4500mg/L Glucose + L-Glutamine + Pyruvate</td>
<td>was supplied by Gibco™ and supplemented with 5% (v/v) Foetal Calf Serum (FCS), 2 mM L-glutamine, and 25 µg/ml Gentamicin</td>
</tr>
<tr>
<td>Alpha Minimal Essential Medium + Ribonucleosides + Deoxyribonucleosides</td>
<td>was supplied by Gibco™ and supplemented with 10% (v/v) Foetal Calf Serum (FCS), 2 mM L-glutamine, and 25 µg/ml Gentamicin</td>
</tr>
<tr>
<td>HAM-F12 Medium + pyridoxine + NaHCO₃ + L-Glutamine</td>
<td>was supplied by Gibco™ and supplemented with 5% (v/v) Foetal Calf Serum (FCS), 2mM L-glutamine, and 25 µg/ml Gentamicin</td>
</tr>
<tr>
<td>RPMI 1640 Medium + L-Glutamine + 25mM HEPES</td>
<td>was supplied by Gibco™ and supplemented with 5% (v/v) Foetal Calf Serum (FCS), 2 mM L-glutamine, and 25 µg/ml Gentamicin</td>
</tr>
</tbody>
</table>
### 2.2.2 General Solutions

**Table 2.3:** Solutions used in this study

<table>
<thead>
<tr>
<th><strong>Solutions</strong></th>
<th><strong>Composition</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Mini-prep Solution I</td>
<td>50mM glucose, 25mM Tris-Cl, 10mM EDTA, pH 8.8</td>
</tr>
<tr>
<td>Mini-prep Solution II</td>
<td>0.2M NaOH, 1% SDS</td>
</tr>
<tr>
<td>Mini-prep Solution III</td>
<td>3M potassium acetate, 5M glacial acetic acid</td>
</tr>
<tr>
<td>PBS</td>
<td>137 mM NaCl, 10 mM Phosphate, 2.7 mM KCl, pH 7.4</td>
</tr>
<tr>
<td>SDS-PAGE gel loading buffer (1x)</td>
<td>50mM Tris-Cl pH 6.8, 100mM Dithiothreitol, 2% SDS, 0.1% Bromophenol blue, 10% glycerol</td>
</tr>
<tr>
<td>Agarose gel loading buffer (6x)</td>
<td>0.02% Bromophenol blue, 0.02% Xylenecyanol FF, 30%, Glycerol in H2O</td>
</tr>
<tr>
<td>Cytomix (for <em>T. gondii</em> transfection)</td>
<td>120mM KCl, 0.15mM CaCl2, 10mM K2HPO4/KH2PO4, 25mM Hepes, 5mM MgCl2, pH 7.6, 5mM ATP, 5mM Glutathione</td>
</tr>
<tr>
<td>Tris/Acetate/EDTA (TAE)</td>
<td>0.04M Tris-Acetate, 0.001M EDTA, pH 8.0</td>
</tr>
<tr>
<td>SDS-PAGE running buffer</td>
<td>25mM Tris, 250mM Glycine, pH 8.3, 0.1% SDS</td>
</tr>
<tr>
<td>Semi-dry transfer buffer</td>
<td>2.5mM Tris, 19.2mM Glycine, 20 % Methanol</td>
</tr>
<tr>
<td>Tris EDTA (TE) 10x</td>
<td>100 mM Tris-HCl, pH 7.5; 10 mM EDTA, pH 8.0</td>
</tr>
</tbody>
</table>

### 2.3 Cell lines and microbiological strains

#### 2.3.1 Bacteria

**Table 2.4:** Genotypes of *Escherichia coli* used

<table>
<thead>
<tr>
<th>Strain name</th>
<th>genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>XL1-Blue</td>
<td>F’::Tn10 proA B’ lacIΔ(lacZ)M15/recA1 endA1 gyrA96(Nalr) thi hsdR17 (r(c m(c)) glnV44 relA1 lac</td>
</tr>
<tr>
<td>BL21</td>
<td>F ompT gal [dem] [lon] hsdS(rH mH) λ(DE3)</td>
</tr>
</tbody>
</table>
2.3.2 *Pichia pastoris*

Table 2.5: Strains of *P. pastoris* used

<table>
<thead>
<tr>
<th>Strain name</th>
<th>genotype</th>
<th>phenotye</th>
</tr>
</thead>
<tbody>
<tr>
<td>GS115</td>
<td>his4</td>
<td>MUT⁺</td>
</tr>
</tbody>
</table>

2.3.3 Mammalian cells

Human Foreskin Fibroblasts (HFF), the African Green Monkey Kidney Cell line (Vero), and the Chinese Hamster Ovary (CHO) cell line “lec2” were obtained from the American Type Culture Collection (ATCC). CHO-K1, CHO-A745, CHO-B618 and CHO-D677 were a kind gift from Dr. Fiona Tomley (Institute for Animal Health, Berkshire, UK).

2.3.4 *Toxoplasma gondii* strains

Table 2.6: Strains of *Toxoplasma gondii* used

| Strain Name   | Description                                                                 |
|---------------|                                                                            |
| RHΔhxgprt     | Virulent strain with disruption of *HXGPRT* gene (Donald et al, 1996)       |

2.4 Culture conditions

2.4.1 Bacterial culture

Bacteria cultures were grown in LB Broth at 37 °C or on plates of LB agar. Ampicillin was used at a concentration of 100 µg/ml. Kanamycin was used at a concentration of 30µg/ml. Glycerol stocks of bacteria strains were stored at -80 °C in LB Broth with 15% (v/v) glycerol.

2.4.2 *Pichia pastoris* culture

Culture of *P. pastoris* was performed as indicated in the manufacturers instructions (Invitrogen).
2.4.3 Mammalian cell culture

HFF and Vero cells were grown as monolayers in supplemented DMEM. CHO cell lines were grown in conditions as indicated by the ATCC: CHO-lec2 cells were cultured in supplemented Alpha minimum essential medium, other CHO cells were grown in supplemented HAM-F12K medium. C6 rat glioma cells were propagated in supplemented RPMI. Cells were split using Trypsin (Gibco).

2.4.4 Parasite propagation

*T. gondii* tachyzoites were grown in Vero or HFF cell cultures. Selection and cloning of stably transfected parasites took place in HFF cell cultures, with medium supplemented with appropriate drug (25 μg/ml mycophenolic acid (MPA), 50 μg/ml Xanthine, 1 μM Pyrimethamine, 20 μM chloramphenicol).

2.5 Transformations and transfections

2.5.1 *E. coli* transformation

Competent *E. coli* were prepared using the protocol of Inoue *et al* (Inoue et al, 1990). Transformations were performed by mixing competent cells with DNA, followed by incubation on ice for 10 minutes, 1 minute of heat-shock at 42°C, and a further 5 minutes on ice, followed by plating on LB-Agar supplemented with an appropriate antibiotic.

2.5.2 *Pichia pastoris* transformation

Transformation of *P. pastoris* was performed by electroporation as indicated in the manufacturers instructions (Invitrogen).
2.5.3 *T. gondii* transfection

For *T. gondii* transfection, $5 \times 10^7$ extracellular parasites freshly egressed from host cells were used for each transfection. Parasites were centrifuged at 300 g for 10 mins, then resuspended in 700 µl cytomix per transfection and mixed with 50 µg of linearised plasmid DNA for stable transfections or 100 µg of supercoiled plasmid DNA for transient transfections. For random integration, restriction enzyme-mediated integration (REMI) was employed by adding 50-100 units of restriction enzyme to the transfection mix. Parasite/DNA mix was then placed in a 4 mm electroporation cuvette and electroporated with the following settings: Voltage: 2000 V, Resistance, 50 Ω, and Capacitance 25 µF (Soldati & Boothroyd, 1993). After electroporation, the transfection mix was added to HFF cells.

2.6 Plasmid construction

This is described in the Materials and Methods of the respective publications together with the primers used.

2.6.1 Agarose gel electrophoresis

0.5 – 2% agarose gels were made by dissolving agarose (Sigma) in heated TAE, and pouring the solution into a gel casting tray (peqLab). Ethidium Bromide (0.5 µg/ml) or SYBR safe (at 0.4x concentration, Molecular Probes™) was added to the gel to stain nucleic acids. Electrophoresis was performed in TAE buffer.

2.6.2 Preparation of nucleic acids

Total RNA was isolated from 1–5 $\times 10^7$ parasites using Trizol reagent (Invitrogen) following the manufacturers instructions. Preparation of cDNA, either using gene specific or general Poly-A primers was performed using Superscript II Reverse Transcriptase (Invitrogen) according to the manufacturer’s instructions.

Mini-preps of plasmid DNA from *E. coli* were performed using the alkaline lysis method (Sambrook *et al.*, 1989). Briefly, cell pellets from a 5 ml culture were resuspended in
100 µl mini-prep Solution I. 200 µl of Solution II was added, and the sample was incubated for 5 minutes at room temperature. 150 µl of Solution III was added, and samples were incubated on ice before centrifugation for 10 minutes at 13000g at 4 °C. Supernatants were then mixed with 2.5 volumes of cold ethanol to precipitate nucleic acids. Following centrifugation for 15 minutes at 13000g at 4 °C and washing with 70 % ethanol, nucleic acid pellets were resuspended in water supplemented with RNase A (0.1 mg/ml). Large preparations of plasmid DNA from *E. coli* were performed using the Nucleobond Maxiprep Kit according to the manufacturer’s instructions.

### 2.6.3 Polymerase chain reaction (PCR)

Reverse transcription reactions were performed using Superscript II Reverse Transcriptase (according to the manufacturer’s instructions) when required to prepare the first strand for cDNA synthesis. Standard analytical PCR reactions were performed with AmpliTaq, using the supplied buffer, with 0.05 units of enzyme per µl of reaction, primers at 0.4 µM, and dNTPs at 0.1 mM each. Template DNA was added to each reaction in the form of plasmid DNA (0.1-1 ng per reaction), genomic DNA (1-5 µl of genomic preparation) or a bacterial colony. The standard reaction temperatures used were an initial 94 °C for 5 mins, followed by 25 cycles of 94 °C for 30s (denaturing step); 55 °C for 30s (annealing step), 72 °C for 1 min/kb of product (elongation step), followed by a final 7 mins at 72 °C. Preparative PCR reactions for cloning were prepared under similar conditions, with the following exceptions: TaKaRa LA Taq was used, an elongation temperature of 68 °C was used, and when using genomic DNA or cDNA as a template, 35 cycles were performed.

### 2.6.4 Ligations

TA cloning of PCR products was performed using either pGEM T-easy (Promega) or TOPO (Invitrogen) cloning kits according to the manufacturer’s instructions. The correct DNA sequences of clones were confirmed by sequencing. For other plasmids, inserts were prepared either by restriction digest of purified PCR products, or by restriction digest of the appropriate plasmid followed by gel purification of the appropriate band. Vectors were prepared by restriction digest, followed by treatment with 200 units of calf intestinal alkaline phosphatase (C.I.P.; QBiogene) at 37 °C for 1 hour, followed by gel purification of the appropriate band.
Ligations were performed with an insert-vector ratio of ~4:1 in a volume of 10 μl using 0.5 units of T4 DNA ligase (Roche) in the supplied buffer, and incubated at room temperature for at least 1 hour or overnight at 4 °C. Ligation reactions were then used to transform E. coli.

2.6.5 Site-directed mutagenesis

Site-directed mutagenesis (SDM) was performed either using the Quikchange II Kit (Stratagene) or using components of the kit bought separately. Complementary sense and antisense primers were designed containing the desired mutation, according to the manufacturer’s instructions. A 50 μl polymerase reaction using 2.5 units of PfuI Turbo or PfuI Ultra (Stratagene) DNA polymerase was performed in the supplied buffer, with 20 ng plasmid DNA, primers at 0.3 μM each, and dNTPs at 0.1 mM each. The reaction temperatures were as follows: an initial 95 °C for 30 s, followed by 12 cycles of 95 °C for 30 s (denaturing step), 55 °C for 1 minute (annealing step); 68 °C for 1 minute per kb of plasmid (elongation step). Following the polymerase reaction, 20 units of DpnI enzyme was added directly to the reaction, followed by incubation at 37 °C for one hour. 1 – 10 μl of the reaction was then used to transform E. coli. The presence of the desired mutation was confirmed by DNA sequencing.

2.7 Sodium Dodecyl Sulphate (SDS) Polyacrylamide Gel (PAGE) Electrophoresis and Western Blotting

SDS-PAGE electrophoresis was performed according to standard methods (Sambrook et al., 1989). Resolving gel (8-15 % acrylamide; 0.1 % SDS; 375 mM Tris, pH 8.8) and stacking gel (5 % acrylamide, 0.1 % SDS, 200mM Tris, pH 6.8) solutions were polymerized using 0.04-0.1 % N,N,N',N'-Tetramethyl-1-,2-diaminomethane (TEMED) and 0.1 % ammonium persulphate (APS). Electrophoresis was performed in SDS-PAGE running buffer. Semi-dry transfer of proteins to nitrocellulose was performed as described (Sambrook et al., 1989), using semi-dry transfer buffer. Blots were blocked in 5% skimmed milk in 0.05% Tween-20 PBS (PBST) for one hour at room temperature, or overnight at 4 °C. Blots were incubated with primary antibody, diluted in blocking solution. Blots were then washed with PBST, and incubated with HRP conjugated secondary antibody, either goat anti-mouse or goat anti-
rabbit, diluted in blocking solution 1:3000. Blots were then developed using ECL reagent (Amersham), and exposed to chemiluminescence film.

When required, blots were stripped with stripping buffer (2% SDS, 7µl/ml 2-β-mercapto-ethanol in PBS) for 45 minutes, washed thoroughly with PBS-T and a further antibody labelling could be performed again. Samples of *T. gondii* which were to be used for western blotting were prepared as follows: freshly egressed *T. gondii* cells were resuspended in culture medium, centrifuged at 300 g for 10 mins, washed once in PBS, and centrifuged again. The resulting parasite pellet was then resuspended in 200 µl of PBS and snap frozen in liquid nitrogen. The suspension was then sonicated three times for ten seconds, with one minute incubation on ice between each sonication, to lyse the parasites. 50 µl of 5 x SDS-PAGE loading buffer was then added to the lysed parasites, and the samples were loaded onto the gel without boiling.
3. Results

3.1. Atomic resolution insight into host cell recognition by *T. gondii*

Earlier work on TgMIC1 and the TgMIC1-4-6 complex had shown that TgMIC1 possesses host cell adhesive properties and the protein was proposed to bind to lactose (Fourmaux et al, 1996; Lourenco et al, 2001). In the amino-terminal part of the protein a duplicated domain has been identified (Fourmaux et al, 1996). These domains were assumed to be TSR-1 domains of divergent nature due to a CXXXCG motif typically present in this type of domain. However, structural studies on TgMIC1 in the laboratory of our collaborator Prof. Stephen Matthews (Imperial College, London) showed that the N-terminal part adopts a completely unrelated novel fold which was termed Microneme Adhesive Repeat (MAR). Carbohydrate microarray analyses performed in the laboratory of Prof. Ten Feizi (Imperial College, London) revealed that the TgMIC1 MAR domains bind to sialic acid. This carbohydrate has been previously shown to be of importance for *T. gondii* invasion in experiments using a CHO cell mutant (lec2) exhibiting reduced surface expression of sialic acid (Monteiro et al, 1998).

In the light of these results the importance of host cell sialylated glycoconjugates for host cell invasion by *T. gondii* has been reassessed in our laboratory. Cell binding assays were used to determine if the interaction of TgMIC1 with sialylated host cell receptors is physiologically relevant and how this compares to earlier reports of lactose binding. Furthermore mutational studies confirmed the critical role of two threonine residues in TgMIC1 for binding to sialic acid. These results together with results presented in section 3.4. constitute the major personal achievements of this Ph.D..
Atomic resolution insight into host cell recognition by *Toxoplasma gondii*

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The obligate intracellular parasite *Toxoplasma gondii*, a member of the phylum Apicomplexa that includes *Plasmodium* spp., is one of the most widespread parasites and the causative agent of toxoplasmosis. Micronemal proteins (MICs) are released onto the parasite surface just before invasion of host cells and play important roles in host cell recognition, attachment and penetration. Here, we report the atomic structure for a key MIC, TgMIC1, and reveal a novel cell-binding motif called the microneme adhesive repeat (MAR). Using glycoarray analyses, we identified a novel interaction with sialylated oligosaccharides that resolves several prevailing misconceptions concerning TgMIC1. Structural studies of various complexes between TgMIC1 and sialylated oligosaccharides provide high-resolution insights into the recognition of sialylated oligosaccharides by a parasite surface protein. We observe that MAR domains exist in tandem repeats, which provide a highly specialized structure for glycan discrimination. Our work uncovers new features of parasite–receptor interactions at the early stages of host cell invasion, which will assist the design of new therapeutic strategies.

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**Introduction**

*Toxoplasma gondii* is a protozoan parasite that is uniquely adapted to infect a wide range of hosts, including virtually all warm-blooded animals and up to 50% of the world’s human population. The primary transmission route in humans is via contact with feces from infected domestic cats or ingestion of undercooked contaminated meat, particularly lamb. Toxoplasmosis causes a variety of disease states in humans, including severe disseminated disease in immunosuppressed individuals due to reactivation of encysted parasites and birth defects in infants, where mothers are exposed during pregnancy (Hill and Dubey, 2002).

Infection is rapidly established in the host by the fast-replicating form of the parasite, the tachyzoite, which can invade an extremely broad range of cell types. Unlike other pathogens that hijack existing host cell uptake pathways, *Toxoplasma* and other apicomplexan parasites, including *Plasmodium*, actively force entry into host cells. The process is initiated by contact with the host cell plasma membrane, followed by reorientation and then the generation of a motive force, which drives penetration into a novel, parasite-induced structure called the parasitophorous vacuole (Carruthers and Boothroyd, 2006). The rapid and smooth transition through these stages requires a highly regulated release of proteins from several parasite organelles, namely micronemes, rhoptries and dense granules (Carruthers and Sibley, 1997). Microneme discharge occurs first and their contents participate in the attachment of parasites to the host cell surface (Carruthers et al, 1999), and the formation of a connection with the parasite actinomyosin system (Jewett and Sibley, 2003), thereby providing the platform from which to drive motility and invasion (Soldati and Meissner, 2004).

One of the first micronemal proteins (MICs) to be discovered in *T. gondii* was MIC1 (TgMIC1), which functions in cell adhesion (Fourmaux et al, 1996). TgMIC1 is a remarkable, multifunctional protein that in addition to binding host cell receptors, interacts with two other microneme proteins (TgMIC4, TgMIC6) (Brecht et al, 2001; Reiss et al, 2001) and is essential for transport of the entire complex through the early secretory pathways (Reiss et al, 2001; Huynh et al, 2004; Saouros et al, 2005). Deletion of the *mic1* gene in *T. gondii* has also confirmed the specific and critical role played by TgMIC1 in host cell attachment and invasion *in vitro* and has provided evidence for its role in virulence *in vivo* (Cerede et al, 2005). Recent studies have shown that a purified TgMIC1 subcomplex is a potent antigen and acts as an effective vaccine in the mouse model (Loureiro et al, 2006). Unlike the battery of other MICs, the sequence of TgMIC1 does not exhibit an obvious likeness to vertebrate adhesive motifs. However, a recent nuclear magnetic resonance (NMR) structure revealed a previously unidentified and novel galectin-like domain within the C-terminus of...
TgMIC1 that promotes proper folding of TgMIC6 and contributes to complex formation (Sauuros et al., 2005).

Although studies have clearly highlighted the importance of MICs in apicomplexan invasion, the finer structural details of host cell recognition remain largely unknown. So far, two main studies have addressed this issue in Plasmodium falciparum: erythrocyte-binding antigen (EBA-175) binds sialic acid (Tolia et al., 2005) and TRAP binds heparin (Tossavainen et al., 2006), although high-resolution information regarding carbohydrate recognition was not forthcoming. Combining atomic resolution studies with data from carbohydrate microarrays we reveal a novel interaction between T. gondii and a variety of sialylated oligosaccharides. The binding mode is attributed to a new family of domains named the micronemal adhesive repeat (MAR) that exists in tandem repeats and provides a highly specialized structure for glycan recognition. Our work presented here addresses many long-standing issues and uncovers new features regarding parasite–receptor interactions in the early stages of host cell invasion. Furthermore, an understanding of the atomic resolution details of how T. gondii invades host cells opens the way to the design of therapeutic strategies.

Results and discussion

The overall structure of cell-binding region of TgMIC1

To resolve the atomic structure of the host cell-binding region from TgMIC1 we expressed the N-terminal 246 amino acids (residues 17–262, hereafter termed TgMIC1-NT) in Escherichia coli fused with thioredoxin to aid disulfide bond formation and solubility. Binding assays revealed that our bacterially and Pichia-produced TgMIC1-NT bound independently to host cells (Figure 1A). The high binding efficiency observed for E. coli-derived material is likely due to the higher purity of this reagent (Figure 1A). Our earlier experiments on the C-terminal domain from TgMIC1 (TgMIC1-CT) had excluded a role in cell binding for this region, (Sauuros et al., 2005); therefore, we can conclude that TgMIC1-NT possesses the cell-binding properties of the full-length TgMIC1.

After subsequent removal of the purification tag, we crystallized the recombinant protein and a selenomethionine (SeMet)-substituted form of TgMIC1-NT, and resolved the structure using the MAD (multiple-wavelength anomalous dispersion) method. The atomic structure for residues 29–259 was solved at a resolution of 1.9 Å (Figure 2A; Table I and

Figure 1 Host cell binding by TgMIC1-NT. (A) Cell binding assays on HFFs were performed using supernatant of P. pastoris cultures expressing TgMIC1, TgMIC1-NT, or TgMIC1MAR2, bacterially produced TgMIC1-NT and PfProfilin, the latter being used as a negative control. Anti-His antibodies are used as the probe for Western blots and the asterisk indicates background from host cells. Samples of input (I), supernatant (S), wash (W) and cell-binding fraction (CB) were run on each gel (see Materials and methods). Molecular weight markers in kDa. The Pichia-produced material is less efficient as it is a crude cell supernatant, whereas that from E. coli is a purified protein. These data show that bacterially produced TgMIC1-NT retains the cell-binding activity of native TgMIC1. Increasing the concentration of the input (I) up to 50 times results in enhanced binding of bacterially produced TgMIC1NT but not of PfProfilin to HFFs. Loading has been adjusted for detection in the linear range. Note: in all cell binding assays, the total volume of the input, supernatant and the wash is 500 µl, whereas the total volume of the cell-bound fraction is 50 µl. (B) Cell binding competition experiments were performed using bacterially produced HisTgMIC1-NT and supernatants of P. pastoris cultures expressing TgMIC1myc. Anti-myc and Anti-His antibodies were used as probes for Western blots. Samples are named as follows: input (I), supernatant (S), wash (W) and cell binding fraction (CB). For the different conditions of competition only the cell-bound fraction is shown. These data confirm that no inhibition of TgMIC1 binding to HFFs is observed in the presence of lactose, galactose or heparin. Note: in all cell binding assays, the total volume of the input, supernatant and the wash is 500 µl, whereas the total volume of the cell-bound fraction is 50 µl.
Supplementary Figure 1). Residues 17–28 and 262 likely exhibit a degree of flexibility and therefore could not be observed in the crystal structure. The new structure reveals a repeated domain consisting of a distorted barrel arrangement of five \(\beta\)-strands, which is flanked on one side by an antiparallel helical bundle comprising one helix from each terminus. Two disulfide bonds, C1–C4 and C5–C7 (namely C45–C85 and C103–C113 in repeat 1 and C193–C203 in repeat 2; Figure 3A), are absolutely conserved between the repeats and stabilize the core structure, one connecting helix \(a_1\) to the \(\beta\)-barrel and the other between strands \(b_3\) and \(b_4\). Although the two repeats (amino-acid residues 16–144 and 145–237) have a sequence identity of 27% and superimpose with a backbone r.m.s.d. of 2.2 Å over 89 residues (Figure 2B), some notable differences are apparent. The first helix and the subsequent loop to strand \(b_1\) are significantly longer in the first repeat and are stabilized by an additional disulfide bond, C53–C61 (Figure 3A). Most strikingly, the second repeat is elaborated at its C-terminus by a short ‘\(\beta\)-finger’ (amino-acid residues 238–256) which is pinned to the main body of MAR2 by a new arrangement of two disulfide bonds C6–C9 and C8–C10 (namely C197–C242 and C236–C252; see Figure 3A) replacing the single connection observed in repeat 1, C6–C8 (namely C107–C143; Figure 3A).

**The MAR domain—a new fold unrelated to thrombospondin type1 repeats**

In the initial characterization of TgMIC1 it was postulated that a tandem arrangement of degenerate thrombospondin type I repeats (TSRs1) was present at the N-terminus (Fourmaux et al, 1996). TRS1s adopt an antiparallel, three-stranded fold comprising alternating stacked layers of tryptophan and arginine residues. Our structure of TgMIC1-NT now enables us to reassess this domain classification and reveals a fold that bears no resemblance to the TSR1s. Moreover, it is unrelated to the classical \(\beta\)-sandwich structure of the prototypic surface antigen, SAG1, from Toxoplasma (He et al, 2002), and to the dimeric arrangement of EBA-175 (Tolia et al, 2005). A search of the DALI database reporting no structural hits confirms this and the fact that the structure of TgMIC1-NT presents a previously unknown protein fold (Holm and Sander, 1995). Based on these observations, we have named this repeat domain the ‘MAR domain’, after micronemal adhesive repeat.

A search against other apicomplexan genomes identified tandem MAR domains in MIC1 from Neospora caninum (Keller et al, 2002) and in three other MIC1-like proteins in T. gondii (Figure 3A), which may help to endow this parasite with the ability to invade a wide range of cell types as well as elements that may correspond to an interaction site, possibly for its partner in the complex MIC4 (Saouros et al, 2005).
evade host immune responses. MIC3 proteins from *Eimeria tenella*, the cause of coccidiosis in poultry, contain between four and seven consecutive MAR1 domains constructed from five distinct MAR1 sequences (Labbe et al., 2005) (Figure 3A). A model of an uninterrupted stretch of MAR1 domains, as in EtMIC3, generated using our structure of TgMIC1-NT, gives rise to a stalk structure comprising a left-handed helical axis with 70° rotations and 11 Å translations relating adjacent MARs (Figure 3B). This arrangement would project from the parasite surface, presenting an array of MAR domains that could provide increased cell-binding avidity (Supplementary Figure 2). Future studies will be aimed at unveiling other parasite surface proteins that possess members of the MAR family.

**The MAR domain—a novel carbohydrate-binding domain**

A long-standing question is how *T. gondii* can infect and replicate within all nucleated cells. The broad host range suggests that complementary receptors exist on a wide variety of host cell types. Carbohydrate recognition and discrimination provide excellent means to facilitate such interactions and often play an important role in early recognition events by invasive pathogens. Although carbohydrate-binding properties have been described for microneme proteins from *Toxoplasma* (Cerede et al., 2002; Harper et al., 2004), studies on the nature of these interactions have not been forthcoming. Monteiro et al. (1998) showed that sialic acid plays a role in host cell invasion, but the identity of the parasite ligand has been the subject of much speculation. Indirect evidence exists for a lactose-binding activity for TgMIC1 and the TgMIC1–TgMIC4 subcomplex (Lourenco et al., 2001, 2006). However, cell binding inhibition (Figure 1B) and NMR titration experiments performed in the present study failed to detect lactose recognition by TgMIC1.

To reassess the carbohydrate-binding properties of TgMIC1, microarray analyses (Campanero-Rhodes et al., 2006; Palma et al., 2006) were carried out using the fusion proteins TgMIC1-NT and TgMIC1-CT, and lipid-linked oligosaccharide probes (Feizi and Chai, 2004), as described. The microarrays encompassed a panel of >200 oligosaccharide probes representing diverse mammalian glycan sequences and their analogues, as well as some derived from fungal and bacterial polysaccharides (Palma et al., 2006) (Supplementary Table 1).

### Table 1 Data collection, phasing and refinement statistics (also see Supplementary Figure 1)

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<th>SeMet Peak</th>
<th>SeMet Inflection</th>
<th>SeMet Remote</th>
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<th>2,3-sialyl-N-acetyllactosamine</th>
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<td>66.3, 172.6</td>
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<td>66.1, 172.7</td>
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<td>18.0–1.9</td>
<td>18.0–1.9</td>
<td>18.0–1.9</td>
<td>20.0–2.0</td>
<td>18.0–2.3</td>
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<td>90 740</td>
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<td>10 069</td>
<td>10 070</td>
<td>49 275</td>
<td>17 796</td>
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<th>2,3-sialyl-N-acetyllactosamine soak</th>
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<td>17.5–1.9</td>
<td>17.5–1.9</td>
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<td>16 806</td>
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<td>10.2 (29)</td>
<td>10.2 (29.1)</td>
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<tr>
<td>Completeness (%)</td>
<td>96.6 (76.2)</td>
<td>99.9 (100)</td>
<td>99.9 (100)</td>
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</table>

<sup>a</sup>Values in parentheses correspond to the highest-resolution shell.
<sup>b</sup>R<sub>free</sub> = \[ \sum \frac{||F(h)| - \langle|F(h)|\rangle||}{\sum |F(h)|} \] where |F(h)| is the rth measurement.
<sup>c</sup>R-factor = \[ \sum \frac{||F(h)| - |F(h)|_{calc}||}{\sum |F(h)|} \] if |F(h)| is calculated in the same way as the R-factor, using only 5% of reflections randomly selected to be excluded from the refinement.
Figure 3  MICs from apicomplexan parasites contain the TgMIC1 repeat. (A) Structure-based sequence alignment of MAR1 and MAR2 domains from other MICs. Including TgMIC1 (MAR1 amino-acid residues 32–144, MAR2 amino-acid residues 145–263), two of the three MIC1-like proteins from *T. gondii* (MAR1 amino-acid residues 114–222, MAR2 amino-acid residues 223–336 in TgMIC1a and MAR1 amino-acid residues 114–222, MAR2 amino-acid residues 223–335 in TgMIC1b), NcMIC1 (MAR1 amino-acid residues 30–142, MAR2 amino-acid residues 143–261) and EtMIC3 (MAR1 amino-acid residues 42–149, MAR1a amino-acid residues 150–274, MAR1b amino-acid residues 291–425, MAR1c amino-acid residues 36–158 and MAR1d amino-acid residues 180–280). For clarity, the third MIC1-like protein, TgMIC1c, from *T. gondii*, has been omitted. Conserved residues are shaded in blue. Cysteines and disulfide bond connectivities are highlighted in orange. Cis-proline within the \textit{232NPPL235} motif is shown in red. Secondary structure elements are indicated above the sequences. (B) A schematic representation of a model for the seven sequential MAR1 domains from EtMIC3 is shown in two orientations (left, perpendicular to the helical axis and right, along the helical axis).
The C-terminal galectin-like domain, TgMIC1-CT, neither showed binding to galactose-terminating glycans in the array, consistent with our early NMR evidence (Saucos et al., 2005), nor was there binding to any of the other probes in the microarray (results not shown). In contrast, significant binding signals with fluorescence intensities between 150 and approximately 8000 were elicited by TgMIC1-NT and were observed only among terminally sialylated structures. (Figure 4 and Supplementary Table 1). All the non-sialylated structures tested had numerical scores below 150 (Supplementary Table 1).

More than 40 out of the 69 sialylated probes arrayed gave numerical binding scores greater than 150 with TgMIC1-NT; among them were several N- and O-glycans, and gangliosides, in groups D, G and F, respectively, and others representing sialylated capping structures on backbones of mammalian glycoconjugates in groups A, B, C and E (Figure 4). Where close comparisons could be made (Table II), a mild preference is apparent for the Neu5Ac group in MAR2 domains D, G and F, respectively, and others represented Table 1). The non-sialylated structures tested had numerical scores below 150 (Supplementary Table 1).

Results from the carbohydrate microarrays reveal that a variety of sialyl oligosaccharide sequences, as found on glycoproteins and glycolipids, are recognized with specific preferences for sialic acid linkage position and some discrimination of oligosaccharide backbone sequences. Interestingly, the most potent binders were branched carbohydrates having two or more terminal sialic acids, raising the possibility that the tandem MAR repeat recognizes specific bidentate ligands. Optimal binding responses are observed when sialic acid termini are separated by five to eight carbohydrate units, which suggests that this separation could be sufficient to span both MAR domains of one TgMIC1 molecule (Table II).

TgMIC1 presents a fixed arrangement of two sialic acid-binding sites

Although the key sialic acid recognizing residues are conserved and located in structurally identical positions in both MAR1 and MAR2 domains (Figure 3A), and despite exhaustive crystal soaking experiments, no oligosaccharides were found to be bound in MAR1. Instead, an acetate molecule interacts with the equivalent threonine in MAR1 (Thr126) in an manner identical to the carboxyl group of sialic acid in MAR2, suggesting that MAR1 is able to recognize sialyl oligosaccharides, albeit perhaps with a lower affinity (Figure 5B). The most likely reason for the absence of glycan in MAR1 is the extensive crystal contacts in which this region is involved. The binding site in MAR1 is very close to a two-fold symmetry axis and residues Thr126, Arg127 and Gln129 are involved in crystal contacts. The presence of the glycan in MAR2, and its absence in MAR1, were confirmed with a simulated annealing omit map. To investigate the potential 2:1 glycan:protein stoichiometry, and to provide site-specific information, we performed a NMR titration experiment using $^{15}$N,13C-Ala/Thr-labelled TgMIC1-NT in the presence of various sialyl carbohydrates. Using a combination of triple-resonance data and information from the site-specific labelling, amide chemical shifts for the two key threonine residues, namely $^{15}$HATR$^{27}$ and $^{218}$HYTE$^{221}$, were unambiguously assigned in MAR1 and MAR2, respectively (Figure 7A). Significant amide chemical shift changes are observed for both threonines in the presence of either $\alpha$-2,3-sialyl-2-acetyllactosamine or $\alpha$-2,6-sialyl-N-acetylactosamine, confirming that both MAR domains are active in binding sialic acid (Figure 7A).

To test the importance of both carbohydrate-binding sites on function, we performed site-directed mutagenesis and assessed their respective cell-binding capability (Figure 7B). As active site threonine residues make specific side-chain and main-chain contacts to the sialyl carbohydrate group, we assumed that their disruption would have a major impact on binding. As predicted, the double mutant T126A/T220A-TgMIC1 exhibited no observable binding to host cells. Additionally, both single mutants, namely T126A-TgMIC1 and T220A-TgMIC1, were also defective in binding, thus confirming the importance of both binding sites. To assess the role of the ‘β-finger’ extension in MAR2 (Figure 2C) in cell binding, an experiment was also conducted for a mutant lacking...
amino-acid residues 238–262. No effect of this truncation on cell binding was observed (Figure 7B). Instead, expression of the mutant in *T. gondii* followed by immunofluorescence studies suggests that this part of the molecule is important for binding to its partner protein in the complex TgMIC4 (data not shown).

Figure 4 Carbohydrate microarray data on sialyl glycan binding by TgMIC1-NT. Numerical scores are shown of the binding signals, means of duplicate spots at 7 fmol/spot (with error bars) for the 58 sialyl oligosaccharide probes examined. Sixty-nine positions are shown, as 11 of the probes were printed more than once (Supplementary Table 1). Selected sialo-oligosaccharide sequences are annotated, with designations of Neu5Acα-2,3-gal linkage as pink; Neu5Acα-2,6Gal, blue; NeuGcα-2-3Gal, green and Neu5Acα-2,8 linkage yellow. The scores for the non-sialylated probes in the microarray are not shown. These are provided in Supplementary Table 1 (positions 70–218).
importance of this interaction is highlighted by the observa-
tions provided by structural studies. The structural studies provide the first insights into the interaction between a key microneme protein complex and the host. Not only do we identify an interaction between the N-terminal portion of TgMIC1, TgMIC4 and TgMIC6 (Saouros et al., 2007), but we also provide the atomic resolution basis for recognition. The structural studies emphasize the importance of the MAR domains in penetra-
tion.

A model for glycan recognition by TgMIC1

It is well accepted that the binding of MICs to host cells provides a ‘molecular bridge’ to the parasite and initiates invasion. Few detailed structures of microneme proteins are known and their interactions with the host are especially poorly characterized. Microarray experiments combined with structural studies provide the first insights into the interaction between a key microneme protein complex and the host. Not only do we identify an interaction between the N-terminal region of TgMIC1 and sialylated host cell ligands, but we also provide the atomic resolution basis for recognition. The importance of this interaction is highlighted by the observation that the ability of T. gondii to invade lec2, a CHO mutant deficient in sialic acid, is reduced to about 30% (Monteiro et al., 1998). In this study, we extend these data by performing cell invasion assays in either the presence of soluble carbohydrates or using cells pretreated with neuraminidase. Compared to controls, both conditions severely reduced the levels of parasite internalization (Figure 7C and D), thus confirming the importance of sialic acid recognition for efficient invasion. The specific contribution made by the MAR domains has been established by studies reporting the invasion by mic1KOs to be reduced by ~50% compared to wild-type (Cerede et al., 2005), and by our earlier work ruling out carbohydrate and cell-binding activities for the C-terminal portion of TgMIC1, TgMIC4 and TgMIC6 (Saouros et al., 2005). Furthermore, antibodies directed against the MAR domains from EtMIC3 from E. tenella (Figure 3) inhibit invasion and further development (Labbe et al., 2005), thus emphasizing the importance of the MAR domains in penetration.

Sialic acids are widely distributed in animal tissues, especially in glycoproteins and gangliosides, and have been shown to play an important role in several protozoa–host cell interactions. A prominent example is the erythrocyte-binding antigen (EBA-175) from P. falciparum, which recognizes the heavily sialylated receptor glycoporphin A during invasion by the malarial parasite (Tolia et al., 2005). Although

### Table II

Comparison of TgMIC1 binding signals elicited by selected sialyl probes in the carbohydrate microarray

<table>
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<th>Name (position in Figure 4)</th>
<th>Sequence</th>
<th>Fluorescence intensity at 7 fmol per spot</th>
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<td>NeuAc2-3Galβ-4GlcNAc</td>
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<tr>
<td>SA (6’) LaeNac (6)</td>
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<tr>
<td>Sialyl Leα (21)</td>
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<td>6’ SU-Sialyl Leα (19)</td>
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<tr>
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</table>
sialyl lactose-binding sites were identified in EBA-175, high-resolution insight into glycan recognition was not forthcoming. A model was proposed in which the dimeric receptor-binding domain assembles around a glycophorin A dimer, with carbohydrate binding within the channels. Our structural and binding experiments reveal that TgMIC1 possesses two sialic acid-binding sites uniquely arranged in tandemly repeated MAR domains. These lie on one side of the molecule and,

Figure 5 Structure of TgMIC-NT in complex with sialyl oligosaccharides. (A) Simulated annealing (F_o–F_c) OMIT map contoured at 3σ (left) and 2σ levels (right) for the TgMIC-NT–glycan complex showing the unambiguous orientation of the sialic acid moiety and the position of the galactose unit of α-2,3-sialyl-N-acetyllactosamine. The omit map was calculated with the glycan omitted; stick models of key side chains (green) and sialic acid (Magenta) are overlaid on the map. (B) Structure of α-2,3-sialyl-N-acetyllactosamine in complex with the MAR2 domain from TgMIC1-NT. Ribbon representation of MAR2 is shown in green. Key interacting side chains and the oligosaccharide are shown as stick representations. Oxygen and nitrogen atoms participating in hydrogen bonds are colored in red and blue, respectively. Note: the structure of the α-2,6-sialyl-N-acetyllactosamine complex is shown in Supplementary Figure 1. (C) Structure of the MAR1 domain from TgMIC1-NT shows the position of the acetate molecules within the active site. Ribbon representation of MAR1 is shown in blue. Key interacting side chains and the acetate molecule are shown as stick representations. (D) Ribbon representation of TgMIC1 showing the separation and relative geometries of two sialic acid-binding sites in MAR1 and MAR2.
Figure 6 Sialic acid competes with TgMIC1 cell-binding activity. Cell binding competition experiments with sialic acid were performed on HFFs using supernatants of a P. pastoris culture expressing TgMIC1myc (top), bacterially produced HisTgMIC1-NT (middle) or a P. pastoris culture expressing TgMIC1-NTmyc (bottom). Anti-myc and anti-His antibodies were used as the probe for Western blots. Anti-tubulin antibodies were used as a control for equal amount of cell material used in the assay. For the competition experiments with sialic acid, only the cell-bound fractions are shown. In case of pretreatment of HFFs with neuraminidase, samples of input (I), supernatant (S), wash (W) and cell-binding fraction (CB) were run on each gel. These data confirm that TgMIC1 binds to sialic acid exposed receptors on HFF cells. Note: in all cell binding assays, the total volume of the input, supernatant and the wash is 500 μl, whereas the total volume of the cell-bound fraction is 50 μl. NANA, N-acetylneuraminic acid.

together with their fixed separation and relative geometry, are predicted to bind cognate carbohydrate receptors with high specificity and affinity (Figure 5C). Intriguingly, some of the most potent ligands were multivalent carbohydrates possessing two or more sialic acid units, such as those on gangliosides or polysialic acid. Gangliosides belong to a class of glycosphingolipids that are found in abundance within the membranes of neuronal cells (Karlsson, 1998) and the glycosphingolipids that are found in abundance within the membranes of neuronal cells (Karlsson, 1998) and the gangliosides or polysialic acid. Gangliosides belong to a class of sialic acid residues 238–262). Similarly, the fragment obtained from pROP1mycMIC1NT with primers 5′-GCGCTA GGTGGGCGCAAGACATATGAGAAG-3′ (MIC1-1_672) and 5′-CCGG GCCGGCGCCGCAAGACATGGGCGTCAGCGGATCC-3′ (TgMIC1-17_1717) was digested with NotI and cloned back into pPICZa-TgMIC1NT, resulting in pPICZa-TgMIC1NTAChern (amino acid residues 238–262). Similarly, the fragments obtained from pROP1mycMIC1NT with primers 5′-CCGGTCTCAGGCAAGACATATGGGCGTCAGCGGATCC-3′ (TgMIC1-17_1717) was digested with NotI and cloned back into pPICZa-TgMIC1NT, resulting in pPICZa-TgMIC1NTAChern.

Materials and methods

**Cloning, expression and purification from E. coli**

TgMIC1-NT, spanning residues 17–262 in TgMIC1 (the first 16 residues represent the signal peptide), was expressed using the pET32Xa/LIC plasmid (Novagen) in E. coli Origami (DE3) (Strategene) at 28°C (Saurores et al, 2007). Protein expression was induced with 800 μM isopropyl β-D-thiogalactopyranoside. The hexahistidine-thioredoxin-MIC1-NT fusion protein was purified using nickel-

**Pichia pastoris expression**

*P. pastoris* transformation and expression was performed using the *Pichia* expression kit (Invitrogen) according to the supplied protocols, and all yeast strains were maintained on yeast extract-peptone–dextrose (YPD)-rich media. Transformation of the supplied host strain GS115 was performed by electroporation following linearization with *PmeI* for all pPICZa-based vectors. Selection of transformants was then performed on YPD–zeocin (100 μg/ml). Expression was performed using BMGY and BMMY media according to the manufacturer’s instructions.

**Cell binding assays**

These were performed as described previously (Brecht et al, 2001). Briefly, confluent monolayers of human foreskin fibroblasts (HFF) cells, grown in 12-well plates, were blocked for 1 h at 4°C with 1% bovine serum albumin (BSA) in cold PBS, 1 mM CaCl2 and 0.5 mM MgCl2 (CM-PBS). Excess BSA was removed by two 5-min washes with ice-cold CM-PBS. The proteins to be assayed were then added either in the form of *P. pastoris* culture supernatant (0.25 μg), or bacterially produced (0.25 μg), in combination or not with different concentrations of competitors (sialic acid buffered to pH 6.8, lactose, galactose, heparin; all purchased from Sigma-Aldrich) and diluted in cold CM-PBS to a total volume of 500 μl. After incubation at 4°C for 1 h, the supernatant (S) was removed and the cells were washed four times for 5 min with ice-cold CM-PBS. The cell-bound fraction (CB) was collected by direct addition of 50 μl 1× SDS–PAGE loading buffer with 0.1 M dithiothreitol. In some cases, before blocking, HFF cells were pretreated with 66 μM/ml n-2,3,6,8- **Vibrio cholerae** neuraminidase (Calbiochem) in RPMI1640, 25 mM HEPES, 1-glutamine (Gibco) for 1 h at 37°C in a total reaction volume of 1 ml.

**Cell invasion assays in the presence of carbohydrate inhibitors**

Confluent monolayers of HFF cells on glass coverslips in 12-well plates were washed once briefly in DMEM (Gibco) and incubated for 15 min only with 250 μl DMEM (control) or with 250 μl DMEM containing different double concentrated competitors (sialic acid nitritoltriacetic acid HISBind resin (Novagen) and cleaved with Factor Xa (Invitrogen). The cleaved protein was reapplied to the same column and pure MIC1-N-terminus was recovered in the flow-through. The protein was concentrated to ~10 mg/ml and stored at 4 or −20°C in 1 mM CaCl2, 100 mM NaCl and 50 mM Tris–Cl pH 8.0. SeMet-labelled TgMIC1-NT was expressed in minimal media in *E. coli* Origami (DE3), according to the protocol of Van Duyst et al (1993), purified and concentrated as described above, and stored in small aliquots at −20°C. 15N,13C-labelled samples of TgMIC1-NT were produced in minimal media containing 0.07% 15NH4Cl and 0.2% 13C6-glucose. TgMIC1-NT 15N,13C labelled at Ala and Thr positions was produced for published protocols (Matthews et al, 1993).

**Generation of variants and mutants**

Plasmids pPICZa-TgMIC1TSR1 (called pPICZa-TgMIC1NT) and pROP1mycMIC1TSR1 (called pROP1mycMIC1NT) were obtained as described before (Saurores et al, 2005). C-terminal deletions of these clones were obtained from these plasmids by PCR as follows: the fragment obtained from pPICZa-TgMIC1NT with primers 5′-CGGCTA GGGTTGGGCGCAAGACATATGAGAAG-3′ (MIC1-1_672) and 5′-CCGG GCCGGCGCCGCAAGACATGGGCGTCAGCGGATCC-3′ (TgMIC1-17_1717) was digested with NdeI and NorI and cloned back into pPICZa-TgMIC1NT, resulting in pPICZa-TgMIC1NTAChern (amino acid residues 238–262). Similarly, the fragment obtained from pROP1mycMIC1NT with primers 5′-CCGGTCTCAGGCAAGACATATGGGCGTCAGCGGATCC-3′ (TgMIC1-17_1717) was digested with NotI and cloned back into pROP1mycMIC1NT, resulting in pROP1mycMIC1NTAChern.

Mutants of pPICZa-TgMIC1NT were obtained using the Quick-change kit (Strategene). Primers 5′-CAGTATACGCCGACCGGCGCATGACTAGTC-3′ (TgMIC1-18_1729) and 5′-GACGATATCCTCAGACCGCGGCCTGTTACTG-3′ (TgMIC1-19_1730), as well as primers 5′-GACGACCGCGCATGACTAGTC-3′ (TgMIC1-17_1717) and 5′-CGAATTCCTCCCCATATGGGCGG CGCGTCAGGATC-3′ (TgMIC1-16_1716) were digested with NotI and cloned back into pROP1mycMIC1NT, resulting in pROP1mycMIC1NTAChern.

Toxoplasma gondii, a protozoan parasite that will provide a foundation for further functional study and the design of novel therapeutics for parasitic infections.
Afterwards cells were washed twice with 500 μl DMEM, fixed with 4% paraformaldehyde/0.05% glutaraldehyde for 20 min, followed by a 2-min incubation with 0.1 M glycine in PBS. Fixed cells were blocked in PBS and 2% BSA for 20 min. The cells were then stained with anti-TgSAG1 antibody followed by Alexa 488-conjugated goat anti-mouse IgG (H+L) antibody (Molecular Probes–Invitrogen). Afterwards cells were permeabilized with PBS and 0.2% Triton X-100 for 20 min and blocked again in PBS, 0.2% Triton X-100 and 2% BSA for 20 min. Staining was performed with anti-TgProfilin antibody followed by Alexa 594-conjugated goat anti-rabbit IgG (H+L) antibody (Molecular Probes–Invitrogen). Invasion assays were carried out as triplicates of two independent experiments. According to their staining procedure, numbers of extracellular (attached) and intracellular (invaded) parasites were counted in three microscopic fields. For the untreated control, a total of 200 parasites was counted per microscopic field on average and attachment and invasion were set to 100%. With respect to this untreated control, the percentage of attached and invaded parasites was calculated for each condition.

**Cell invasion assays with neuraminidase treatment**
Confluent monolayers of HHF cells on glass coverslips in 24-well plates were incubated in RPMI1640, 25 mM HEPES and L-glutamine (Gibco) at 37°C, with or without 66 mU V. cholerae neuraminidase (Calbiochem) in a total volume of 500 μl. After 1 h, 500 μl freshly lysed T. gondii RH parasites resuspended in RPMI1640, 25 mM HEPES and L-glutamine (37°C) were added to each well and the plate was centrifuged immediately for 5 min at 480 g. Then invasion was allowed to proceed for another 5 min at 37°C. Afterwards cells were washed two times with 500 μl DMEM, fixed with 4% paraformaldehyde, 0.05% glutaraldehyde for 20 min, followed by 2 min incubation with 0.1 M glycine in PBS. Fixed cells were blocked in PBS, 2% BSA for 20 min. Cells were then stained with anti-TgSAG1 antibody followed by Alexa 488-conjugated goat anti-mouse IgG (H+L) antibody (Molecular Probes–Invitrogen). Afterwards cells were permeabilized with PBS and 0.2% Triton X-100 for 20 min and blocked again in PBS, 0.2% Triton X-100 and 2% BSA for 20 min. Staining was performed with anti-TgProfilin antibody followed by Alexa 594-conjugated goat anti-rabbit IgG (H+L) antibody (Molecular Probes–Invitrogen). Invasion assays were carried out in triplicates of two independent experiments. Numbers of intracellular parasites were counted in three microscopic fields. (D) Cell invasion assays using T. gondii RH parasites after pretreatment of target cells with neuraminidase. Four experiments were carried out in parallel. Numbers of intracellular parasites were counted in five microscopic fields.

**Figure 7** Characterization of the interaction of sialyl oligosaccharides with MAR domains and their involvement in host cell invasion. (A) 1H-15N 2D TROSY-HNCO (top) and 1H-15N 2D TROSY-HSQC (bottom) NMR spectra for 13C,15N-labelled Ala, ThrTgMIC1-NT, in the absence (black) and presence (red) of saturating amounts of an α,2,3-sialyl-N-acetyllactosamine. The assignments of the Thr126 and Thr220 were achieved using standard triple-resonance spectra. (B) Cell binding assays using supernatants of P. pastoris cultures expressing TgMIC1, TgMIC1-NT single (T126A and T220A) mutants, TgMIC1-NT double (T126A/T220A) mutant and a TgMIC1-NT C-terminal truncation mutant, lacking the ‘β-finger’ (amino-acid residues 238–262). Note that all proteins produced in P. pastoris have a C-terminal myc tag. Anti-myc antibodies are used as the probe for Western blots. (C) Cell invasion assays using T. gondii RH parasites in the presence of sialic acid (NANA), lactose and galactose. Invasion assays were carried out in triplicates of two independent experiments. Numbers of intracellular parasites were counted in three microscopic fields.

**Crystallization and data collection**
TgMIC1-NT was crystallized in hanging drops by the vapor-diffusion technique. Crystals shaped as tetragonal bipyramids,
measuring approximately 50 × 50 × 100 μm, were obtained by adding 1 μl of protein to 1 μl of well solution containing 3.6 M ammonium acetate and 100 mM bis-tris-propane (pH 7.6) at 17°C. Crystals were briefly soaked in a cryoprotectant solution containing all the components of the well solution and 25% glycerol, and cryocooled in liquid nitrogen. X-ray data for both native (1.9 A) and SeMet-labelled TgMIC1-NT fusion protein complex in casein/BSA: this contained per 100 μl, 10 μg mouse anti-histidine monoclonal antibody, 10 μg biotinylated goat anti-mouse IgG and 4 μg of TgMIC1-NT or TgMIC1-CT fusion protein. The overlaid pads were washed with 150 mM sodium chloride, 2 mM calcium chloride solution and binding was detected using Alexa Fluor 647-labeled streptavidin (Molecular Probes) and 1 μg per ml casein/BSA. After washing, the dried slides were scanned using a ProScanArray (Perkin-Elmer LAS) and Alexa Fluor 647-binding signals quantified using ScanArrayExpress software (Perkin-Elmer LAS). Microarray data analysis and presentation were carried out using in-house software (M S Stoll, unpublished). The binding signals were glycan dose related overall. The results shown in Figure 4 represent binding at 7 fmol per spot.

Structure solution and refinement
The unbound structure was phased using the multiple anomalous dispersion method with the program SOLVE (Terwilliger and Berendzen, 1999), yielding a mean figure of merit of 0.45 and a Z-score of 8.65. The initial model was built automatically in ARP/WARP (Perrakis et al, 1999). Structures were improved manually using Coot (Emsley and Cowtan, 2004). Refinement and water addition were carried out with multiple cycles of Refmac5 (Winn et al, 2001) and ARP/WARP, ARP/warp, MOLREP and Reffmac5 were used within the CCP4 suite (Bailey, 1994). Fifteen acetate ions, two sodium and chloride molecules and two chloride ions were found in the structure, together with 181 water molecules. Sugar-bound structures were phased by molecular replacement using MOLREP (Vagin and Teplyakov, 1997) and the protein atoms from the unliganded structure as the model. Clear density was observed for the ligand atoms. The structures were rebuilt and refined as described above. Refinement statistics are provided in Table 1. A total of 99.5% of residues are within the allowed regions of the Ramachandran plot. The quality of the structure was assessed using procheck (Laskowski et al, 1993) and whatcheck (Hooft et al, 1996). To confirm the presence of the glycan, a simulated annealing Fc-Fo omit map was calculated by omitting the glycan and acetates occupying the binding sites. The models have been deposited in the Protein Data Bank with accession number 2JH1 (free TgMIC1-NT), 2JH7 (TgMIC1-NT in complex with 2,6-sialyl-N-acetyllactosamine) and 2JHD (TgMIC1-NT in complex with 2,3-sialyl-N-acetyllactosamine). The unbound structure was phased using molecular replacement using MOLREP (Vagin and Teplyakov, 1997) and the protein atoms from the unliganded structure as the model. Clear density was observed for the ligand atoms. The structures were rebuilt and refined as described above. Refinement statistics are provided in Table 1. A total of 99.5% of residues are within the allowed regions of the Ramachandran plot. The quality of the structure was assessed using procheck (Laskowski et al, 1993) and whatcheck (Hooft et al, 1996). To confirm the presence of the glycan, a simulated annealing Fc-Fo omit map was calculated by omitting the glycan and acetates occupying the binding sites. The models have been deposited in the Protein Data Bank with accession number 2JH1 (free TgMIC1-NT), 2JH7 (TgMIC1-NT in complex with 2,6-sialyl-N-acetyllactosamine) and 2JHD (TgMIC1-NT in complex with 2,3-sialyl-N-acetyllactosamine). Figures were drawn using the program MacPyMol (DeLano Scientific). The model for the sialic acid complex showed density for the sialic acid that perfectly matched the sialic acid moiety in the other two sugars. The sialic acid structure was therefore not refined further, as the data were at lower resolution (2.6 Å) than the other two sugar moieties and was deemed not to add any extra information.

Microarray analysis of the binding of TgMIC1-NT fusion protein to diverse oligosaccharide probes
Two hundred and eighteen lipid-linked oligosaccharide probes (Supplementary Table 1) were robotically printed on 16-pad nitrocellulose-coated glass slides (FAST slides, Whatman Ltd) as described (Palma et al, 2006). The number of unique sequences printed was 207, as 11 probes were printed more than once. These are grouped (Figure 4 and Supplementary Table 1) according to their backbone sequences and include numerous mammalian-type carbohydrate sequences: N-glycans (neutral and acidic, high-mannose and complex types), O-glycans and blood group-related sequences (A, B, H, Lewisα, Lewisβ, Lewisα and Lewisβ) on linear or branched backbones, and their sialylated and sulfated analogues, gangliosides, glycosaminoglycans, homo-oligomers of sialic acid and fragments of other polysaccharides, ranging in size from two to 20 monosaccharide units and containing 200–2000 sugar residues. The probes were printed at 2 and 7 fmol per spot, in duplicate, using a non-contact arrayer (Piezorray, Perkin-Elmer LAS, UK), with Cy3 dye included to enable post-array monitoring of the slides. For binding analysis with TgMIC1-NT fusion protein, the arrayed pads were overlaid initially for 1 h with blocking solution containing casein (Fisher) with 1% w/v BSA (Sigma) and 10 mM calcium (casein/BSA). The slides were rinsed and overlaid for 1 h with fusion protein complex in casein/BSA: this contained per 100 μl, 10 mg mouse anti-histidine monoclonal antibody, 10 μg biotinylated goat anti-mouse IgG and 4 μg of TgMIC1-NT or TgMIC1-CT fusion protein. The overlaid pads were washed with 150 mM sodium chloride, 2 mM calcium chloride solution and binding was detected using Alexa Fluor 647-labeled streptavidin (Molecular Probes) and 1 μg per ml casein/BSA. After washing, the dried slides were scanned using a ProScanArray (Perkin-Elmer LAS) and Alexa Fluor 647-binding signals quantified using ScanArrayExpress software (Perkin-Elmer LAS). Microarray data analysis and presentation were carried out using in-house software (M S Stoll, unpublished). The binding signals were glycan dose related overall. The results shown in Figure 4 represent binding at 7 fmol per spot.

NMR mapping of sialic acid-binding sites
For NMR experiments, samples of 15N-labelled TgMIC1-NT, uniformly 15N,13C-labelled TgMIC1-NT or TgMIC1-NT specifically labelled with 15N,13C-labelled Ala and Thr were prepared in 20 mM sodium phosphate buffer pH 7, 100 mM NaCl, 4% Complete® protease inhibitor cocktail (Roche Diagnostics Ltd, UK, prepared according to instructions), 0.03% sodium azide and 0.2 mM 2,2'-dimethyl-2-silapentane-5-sulfonic acid, in 90% H2O, 10% D2O at approximately 500 μl in 0.25 ml. Backbone assignment for Thr220 was completed using standard double- and triple-resonance assignment experiments recorded on uniformly 15N,13C-labelled TgMIC1-NT samples (Sattler et al, 1999). For Thr126, these data were supplemented with HNCO spectra recorded on uniformly15N,13C-labelled Ala and Thr. Four peaks were identified and could be assigned to AA, TA, TT and 124HA. Either x-2,3-sialyl-N-acetyllactosamine or x-2,6-sialyl-N-acetyllactosamine in the same buffer was added in several steps up to a 10-fold molar excess and 2D 1H–15N HSQC spectra were recorded at each stage under identical experimental conditions.

Supplementary data
Supplementary data are available at The EMBO Journal Online (http://www.embojournal.org).

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References

3.2 Structural insights into microneme protein assembly reveal a new mode of EGF domain recognition

The architecture of the TgMIC1-4-6 complex has been partly established previously through conventional knockout of the individual genes showing that TgMIC6 interacts with TgMIC1 which in turn recruits TgMIC4 (Reiss et al, 2001). Structural studies demonstrated the importance of an interaction between TgMIC1-GLD and the membrane proximal TgMIC6-EGF3 encompassing also a downstream stretch of acidic amino acids for assembly and correct trafficking of the TgMIC1-4-6 complex (Saouros et al, 2005). Transfection experiments of TgMIC6 mutants with deletion of individual EGF domains into the mic6ko background suggested that TgMIC6-EGF2 might as well be able to interact with TgMIC1-GLD (Reiss et al, 2001). In this study we have investigated on this interaction and the implications for the stoichiometry and functionality of the complex. While all structural studies were performed by the group of Prof. Stephen Matthews (Imperial College, London), the functional dissection of the TgMIC1-4-6 complex in the parasite constitutes the personal contribution from our laboratory. More details on the individual contributions of the authors can be found in the acknowledgements of the article.
Structural insights into microneme protein assembly reveal a new mode of EGF domain recognition

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The obligate intracellular parasite Toxoplasma gondii, a member of the phylum Apicomplexa that includes Plasmodium spp., is one of the most widespread parasites and the causative agent of toxoplasmosis. Adhesive complexes composed of microneme proteins (MICs) are secreted onto the parasite surface from intracellular stores and fulfill crucial roles in host-cell recognition, attachment and penetration. Here, we report the high-resolution solution structure of a complex between two crucial MICs, TgMIC6 and TgMIC1. Furthermore, we identify two analogous interaction sites within separate epidermal growth factor-like (EGF) domains of TgMIC6—EGF2 and EGF3—and confirm that both interactions are functional for the recognition of host cell receptor in the parasite, using immunofluorescence and invasion assays. The nature of this new mode of recognition of the EGF domain and its abundance in apicomplexan surface proteins suggest a more generalized means of constructing functional assemblies by using EGF domains with highly specific receptor-binding properties.

Keywords: apicomplexa; microneme proteins; invasion; solution structure; EGF

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INTRODUCTION

The intracellular protozoan parasite Toxoplasma gondii is uniquely adapted to infect a wide range of hosts, including virtually all warm-blooded animals and humans. Toxoplasmosis causes a variety of disease states in humans, including severe disseminated disease in immunosuppressed individuals owing to reactivation of encysted parasites and birth defects in infants where mothers are exposed to the parasite for the first time during pregnancy (Hill & Dubey, 2002). T. gondii also causes significant disease and economic loss in the farming industry, principally by inducing abortion and fetal abnormality in sheep (Fusco et al, 2007). Raw and undercooked meat from infected animals represents a principal source of human infection with T. gondii (Dubey, 2004). Around 500 million of the world’s population have been infected with Toxoplasma, and toxoplasmosis is considered to be the third leading cause of death attributed to foodborne illness in the United States.

Toxoplasma and other apicomplexan parasites, including Plasmodium (the agents of malaria), share a set of apical secretory organelles such as micronemes and rhoptries, which release a large number of soluble and membrane proteins onto the parasite’s surface during invasion. Microneme proteins (MICs) are stored in the micronemes before secretion, after which they participate in the attachment of parasites to the host cell surface (Carruthers et al, 1999) and in the formation of a connection with the parasite actinomysin system (Jewett & Sibley, 2003), which drives motility and invasion (Soldati & Meissner, 2004). The integrity of the MIC cargo is controlled by several molecular checkpoints during transport and after secretion, which include conformation-dependent sorting and proteolytic processing events. These processes are particularly well characterized for the TgMIC4–MIC1–MIC6 complex (TgMIC4–1–6) from T. gondii (Reiss et al, 2001; Saouros et al, 2005a).

The TgMIC4–1–6 complex binds tightly to host cell receptors and has a central function in invasion and virulence (Blumenschein et al, 2007). TgMIC6 contains a membrane-spanning domain and a carboxy-terminal cytoplasmic tail encompassing the sorting signal that is essential for the accurate targeting of the complex to the micronemes (Fig 1A). TgMIC6 also contains three extracellular epidermal growth factor-like (EGF) domains, the first of which is proteolytically removed during its transport to the microneme organelles and is not present in mature forms of the complex (Meissner et al, 2002a). In addition, an interaction between the third EGF-like domain of TgMIC6 (TgMIC6-EGF3) and the C-terminal galectin-like domain of
comprises a tandem repeat of a new cell-binding motif called the microneme adhesive repeat (MAR), which has been shown to bind to sialylated oligosaccharides on the host cell surface and recruitment of the third component of the complex, TgMIC4 (Blumenschein et al, 2007). TgMIC4 is also a soluble cell-adhesion protein composed of six apple domains that interacts with TgMIC1 and is processed extracellularly, releasing a 15-kDa C-terminal fragment (Brecht et al, 2001).

Here, we describe a new interaction between TgMIC1 and the second EGF domain from TgMIC6 (TgMIC6-EGF2), and provide a high-resolution basis for assembly of this complex. Experiments based on immunofluorescence studies and host cell invasion assays indicate that both MIC1–MIC6 interactions contribute to the formation of active host cell-binding sites for the invasion of T. gondii. Our study provides a new insight into the mode of interaction between TgMIC1–4–6 and host receptors, as both EGF domains contribute to MIC1 recruitment, recognition of host cell and subsequent invasion.

**RESULTS AND DISCUSSION**

**EGF2 from TgMIC6 interacts independently with TgMIC1**

Although interactions between MICs are of central importance to their integrity and surface presentation, little is known about the high-resolution determinant of complex assembly. For example, the mature extracellular portion of TgMIC6 contains two canonical EGF domains (EGF2 and EGF3; Fig 1A), and although TgMIC6-EGF3 is known to interact with the C-terminal galectin-like domain of TgMIC1 (TgMIC1-GLD; supplementary Fig 1 online; Reiss et al, 2001; Saouros et al, 2005a), no role has been reported for the second EGF domain (TgMIC6-EGF2). To address this issue, the second EGF domain from the extracellular portion of mature TgMIC6 (TgMIC6-EGF2) was produced in *Escherichia coli* and assayed for binding to TgMIC1-GLD by gel filtration (Fig 1B). Examination of the gel filtration profiles showed the co-elution of a protein complex of around 25 kDa molecular weight, corresponding to TgMIC1-GLD with TgMIC6-EGF2. To determine the affinity of this interaction, the binary complex was characterized by using isothermal calorimetry. The binding data for TgMIC1-GLD and TgMIC6-EGF2 followed a standard binding curve. Recorded measurements for enthalpy changes during the titration were fit to a model for a single binding event yielding $K_d = 53 \pm 13$ nM with an observed stoichiometry consistent with a 1:1 interaction (Fig 1C).

**A new and specific mode of EGF domain recognition**

The high-resolution solution structure of the TgMIC1-GLD–TgMIC6-EGF2 complex was solved by using heteronuclear multidimensional nuclear magnetic resonance (NMR) spectroscopy (Fig 2A,B; supplementary Table 1 online). In agreement with the structure of TgMIC1-GLD (Saouros et al, 2005a), this region shows the classic β-sandwich of a galectin-like domain when in complex with TgMIC6-EGF2. The structure of the EGF domain within the complex shows a canonical EGF domain topology (Fig 2B), in which two double-stranded β-hairpins stack in a staggered arrangement that is fixed by the three disulphide bonds present within the core. The two β-hairpins from TgMIC6-EGF2 clamp onto one side of the β-sandwich from TgMIC1-GLD burying an intimate and large surface area at the interface (~1450 Å²), which
Fig 2 | Solution structure of the complex between TgMIC1-GLD and TgMIC6-EGF2. (A) Stereo diagram showing C_n traces representing the ensemble of nuclear magnetic resonance-derived structures (TgMIC1-GLD is shown in orange and TgMIC6-EGF2 is shown in green). (B) Ribbon representation of a representative structure for the TgMIC1-GLD–TgMIC6-EGF2 complex (TgMIC1-GLD is shown in orange and TgMIC6-EGF2 is shown in green). Strand assignments are indicated. The orientation shown on the left is identical to that in (A), whereas the orientation shown on the right represents a 90° rotation about the y axis. (C) Stereo diagram showing an enlarged view of interface I from the TgMIC1-GLD–TgMIC6-EGF2 complex illustrating crucial hydrophobic residues at the interface. (D) Stereo diagram showing an enlarged view of interface II from the TgMIC1-GLD–TgMIC6-EGF2 complex. Residues are numbered for both TgMIC1-GLD and TgMIC6-EGF2 (labelled with quotes). EGF, epidermal growth factor-like; GLD, galectin-like domain; MIC, microneme proteins.
explains the high affinity of the complex. Gap volume index for the complex is 1.1 Å, indicating highly complementary interacting surfaces (Laskowski, 1995).

Although TgMIC1-GLD is in contact with many residues along the length of the TgMIC6-EGF2 sequence, two principal interfacial regions can be delineated. The first comprises Ala 82, Val 84, Ala 98 and Val 100 from \( \beta \)-strands H and I in TgMIC1-GLD, which forms an exposed hydrophobic surface on one of the large \( \beta \)-sheets (strands KBGHI; Fig 2B) and interacts with residues Ile 44, Leu 46 and Val 52 from the underside of the C-terminal \( \beta \)-hairpin from EGF2 (Fig 2C; interface I). A second important point of contact is presented by TgMIC1-GLD at one edge of the \( \beta \)-sandwich (Fig 2D; interface II). In the centre of this region are the hydrophobic residues Phe 49, Val 60 and Tyr 99 from TgMIC1-GLD, which form a binding pocket for Ile 35 from TgMIC6-EGF2 (Fig 2D). Classical antiparallel hydrogen bonding patterns are also observed between main chain atoms of the second \( \beta \)-strand (residues Cys 38, Cys 36 and Tyr 34) from the EGF domain and the edge of the KBGHI \( \beta \)-sheet (Ser 96, Ala 98 and Val 100 from strand I) to form a contiguous seven-stranded sheet.

To provide a complete structural insight into the mode of interaction, the high-resolution solution structure of TgMIC6-EGF2 in its unbound form was solved. Although the absence of principal rearrangements of secondary structure between the free and bound forms (r.m.s.d. of 1.8 and 1.4 Å over 102 and 41 C_α atoms within secondary structure elements of TgMIC1 and TgMIC6, respectively) supports a lock-and-key mode of interaction, several loop regions become more ordered on complex formation. For example, the disordered C terminus of TgMIC1-GLD (residues 128–137) provides a ‘latch’ by folding back over the KBGHI \( \beta \)-sheet and making numerous intermolecular contacts with the EGF domain (supplementary Fig 2 online).

To propose a model for the assembly of mature, full-length forms of TgMIC1 and TgMIC6, we used NMR spectroscopy to...
Establish whether TgMIC1-GLD binds to TgMIC6-EGF3 in the same mode as EGF2. Most of the TgMIC1 amides (>85%) experiencing chemical shift changes in the EGF2 complex are also perturbed when TgMIC1 is bound to EGF3 (Fig 3A). Furthermore, the amides showing the largest chemical shift changes, indicative of principal changes in the chemical environment as a result of binding, are of comparable magnitude in both complexes (supplementary Table II online). We also recorded an isotope 13C-filtered/edited nuclear Overhauser enhancement spectroscopy (NOESY) spectrum on a hybrid 13C–15N-labelled sample and confirmed several predicted intermolecular nuclear Overhauser effects (NOEs) based on the TgMIC1-GLD–TgMIC6-EGF2 complex (supplementary Fig 3 online). Therefore, we conclude that the modes of recognition of the two EGF domains by TgMIC1ΔEGF2-MIC6TY in mic6ko, MIC1-ΔGLD in mic1ko, ΔEGF3-MIC6TY in mic6ko, and MIC1-ΔGLD in mic1ko are nearly identical.

Fig 4 | Functional characterization of the mic6 knock out strain and mic6 knock out parasites complemented with either full-length TgMIC6 or ΔEGF2-MIC6TY and ΔEGF3-MIC6TY mutant proteins. Immunofluorescence assays showing that (A) ΔEGF2-MIC6TY and (B) ΔEGF3-MIC6TY are stably expressed and correctly targeted to the micronemes (anti-TY; green). Transport of TgMIC1 to the micronemes is partly restored in these parasites (anti-MIC1; green), as shown by colocalization with the microneme marker AMA1 (anti-AMA1; red). White arrowheads indicate examples of correct targeting to the micronemes. Note that ΔEGF2-MIC6TY is TY-tagged. A control assay is shown (bottom in A,B) in which MIC1-ΔGLD is unable to interact with either EGF domain from MIC6 and rescue correct microneme localization. TgMIC6 and TgMIC1 are retained in the endoplasmic reticulum/Golgi and dense granules, respectively. A green/magenta version of this figure is shown as supplementary Fig 4 online.

(C) Functional assay comparing various parasite strains for their invasion efficiency using RH-2YFP parasites as an internal standard for parasite fitness and human foreskin fibroblasts as host cells. Invasion data were compared by one-way ANOVA followed by a Newman–Keuls test. Invasion data for ΔEGF2-MIC6/6 and ΔEGF3-MIC6/6 parasites are statistically different from MIC6KO and RH-2YFP parasites, supporting the partial complementation of invasion activity in these mutants. ANOVA, analysis of variance; EGF, epidermal growth factor-like; GLD, galectin-like domain; ko, knock out; MIC, microneme protein; RH-2YFP, RH strain of Toxoplasma gondii transformed with a plasmid expressing yellow fluorescent protein.

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are analogous, consistent with a sequence alignment that shows a conservation of crucial interfacial residues between the two EGF domains (20% identity between TgEGF2 and TgEGF3; Fig 3B).

EGF domains are often found in cell surface proteins where they are involved in protein–protein interactions that promote intercellular signalling, often in multivalent interactions to increase specificity. EGF domains are also present in other apicomplexan MICs and in several malarial surface proteins (Anantharaman et al., 2007). A notable example includes a homologue of TgMIC6 identified in the Neospora caninum genome sequence. The alignment of the EGF domains from these two sequences, together with the alignment of galectin-like domains from TgMIC1 and NcMIC1, shows a high degree of conservation at positions identified as crucial interacting residues in the MIC1–MIC6 complex, suggesting that an equivalent complex is assembled in N. caninum (Fig 3B). Further examples containing tandem repeats of EGF domains include TgMIC3, TgMIC7, TgMIC8 and TgMIC9 (Meissner et al., 2002b), MIC4 from Eimeria, Plasmodium falciparum Pfs25, which is expressed in zygotes and ookinete, and the merozoite surface protein MSP1. Our structure for the TgMIC1-GLD–TgMIC6-EGF2 complex provides the first structural insight into the recognition of the EGF domain at the surface of apicomplexans and might represent a generalized mode of interaction, in which the first \( \beta \)-hairpin of the EGF domain extends an intermolecular \( \beta \)-sheet and the second \( \beta \)-hairpin clamps the surface. It is conceivable that \( \beta \)-sheet-rich domains other than GLDs interact with EGF domains in a similar manner.

**EGF2 and EGF3 promote host cell receptor recognition**

Our observation of two independent TgMIC1 interactions within TgMIC6 has profound consequences for the recognition of host cell receptors, as TgMIC1 is able to discriminate oligosaccharides in a highly specific manner (Blumenschein et al., 2007). To determine the relative importance of the twin TgMIC1-binding sites within TgMIC6, transport of the components of the complex was analysed by immunofluorescence assay in a \( \text{mic} \) knockout (\( \text{ko} \)) strain, as well as in \( \text{mic} \) parasites complemented with constructs expressing full-length TgMIC6, TgMIC6-\( \Delta \)EGF3 or TgMIC6-\( \Delta \)EGF2. It has been shown previously that, in the absence of TgMIC6, TgMIC1 is mis-targeted to the dense granules but that the expression of full-length TgMIC6 in the \( \text{mic} \) background fully rescues the correct targeting of TgMIC1 to the micronemes (Reiss et al., 2001; Saouros et al., 2005a). Complementation of the \( \text{mic} \) with either TgMIC6-\( \Delta \)EGF3 or TgMIC6-\( \Delta \)EGF2 also restores the transport of TgMIC1 to the micronemes (Fig 4A,B), suggesting that both TgMIC6 EGF domains are able to interact independently with TgMIC1 within the parasite. It has recently been shown for TgMIC3 that the minimal requirement for microneme delivery is the presence of its pro-peptide together with any one of the adjacent EGF domains (El Hajj et al., 2008). In other studies, it has been suggested that short \( \alpha \)-helical segments are important for secretory organelle sorting (Dikeakos et al., 2007). Interestingly, our structural data show the presence of an \( \alpha \)-helix within the amino terminus of TgMIC6-EGF2, which is immediately adjacent to the predicted TgMIC6-EGF1 cleavage site (Fig 1A); therefore, the subsequent exposure of the helix by the proteolytic removal of TgMIC6-EGF1 could reflect a biological signal that indicates the correct assembly of the complex and, in turn, allows correct targeting.

To determine whether the mutant complexes in the TgMIC6-\( \Delta \)EGF3 and TgMIC6-\( \Delta \)EGF2 parasites are functional in invasion, we characterized the relative invasion efficiency of the \( \text{mic} \) and the various complemented strains. The host cell invasion efficiency of the \( \text{mic} \) strain was reduced to around 50% of the level of the parental RH strain (Fig 4C). Invasion efficiency was partly restored when the \( \text{mic} \) strain was complemented with TgMIC6, TgMIC6-\( \Delta \)EGF3 or TgMIC6-\( \Delta \)EGF2, providing evidence that both EGF domains allow the presentation of TgMIC1 and recognition of host cell receptors for invasion (Fig 4C). Microneme targeting and subsequent invasion by the mutant parasites are not as efficient as with full-length TgMIC6, which most likely reflects the perturbed stoichiometry of the complex (Fig 4).

**Speculation**

Fig 5 shows a model of the relative arrangement of the two EGF domains based on a structural alignment with the tandem EGF domains from MSP1. Both TgMIC1-binding sites lie on opposite sides of the complex and are available for interaction, which projects the host cell-binding MAR domains of TgMIC1 and the associated TgMIC4 into the extracellular milieu. Previous results

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**Fig 5** A new model for the architecture of the TgMIC4–1–6 complex. A schematic representation of a model for the TgMIC4–1–6 complex illustrating the relative location of the domains and host cell-binding sites. The sialic acid-containing receptor(s) are shown as pink space-filling spheres. Colour code: yellow, apple domains; purple, microneme adhesive repeat (MAR) domain; orange, galectin-like domain (GLD); green and blue, second and third epidermal growth factor-like (EGF) domains; grey, transmembrane (TM); and red cylinder, acidic region. The six apple domains of TgMIC4 are represented as yellow circles, as no structural information is available. MIC, microneme protein.
from carbohydrate microarray experiments have shown that the most potent binders of TgMIC1 were branched carbohydrates having two or more terminal sialic acids, which raised the possibility that tandem MAR regions provide highly specialized ligands for recognizing bidentate receptors (Blumenschein et al., 2007). Our new discovery extends this model for TgMIC4–1–6 complex function in that four receptor-binding sites could be presented through two branches of TgMIC1 molecules bound to one TgMIC6. This subunit multivalency is further reminiscent of the erythrocyte-binding antigen (EBA-175) from P. falciparum (Blumenschein et al., 2007; Hager & Carruthers, 2008), which is dimeric and comprises tandem Duffy-binding ligand domains that recognize several sialyl glycans (Tolia et al., 2005).

METHODS
Recombinant TgMIC1-GLD, TgMIC6-EGF2 (residues 87–147) and TgMIC6-EGF3 (residues 141–209) were expressed and purified as described previously (Saouros et al., 2005a,b).

*T. gondii mic6* parasites, as well as the *mic6*-complemented stable parasite strains MIG6TY, MIC6-AEGF2 and MIC6-EGF3, were obtained as described previously (Reiss et al., 2001). Indirect immunofluorescence assays were used to probe the transport of TgMIC6 and TgMIC1. Comparison of the various *T. gondii* strains for invasion efficiency was carried out using the RH strain transformed with a plasmid expressing yellow fluorescent protein (RH-2YFP) as the internal standard.

The interaction between TgMIC1-GLD and either TgMIC6-EGF2 or TgMIC6-EGF3 was probed using two-dimensional 1H–15N heteronuclear single quantum coherence (HSQC) NMR mapping experiments, gel filtration chromatography and isotothermal titration calorimetry.

For the structural determination, NMR spectra on the complex were recorded on hybrid labelled samples of 15N, 13C- or 14N, 12C-TgMIC1-GLD (residues 320–456 in the full-length TgMIC1 sequence) and 14N, 12C- or 15N, 13C-TgMIC6-EGF2 (residues 87–147 in the full-length TgMIC6 sequence). Backbone and side-chain assignments were completed using standard methodology (Sattler et al., 1999). Intermolecular NOE identification was aided by filtered (12C,14N)-H-NOESY–13C-HSQC experiments (Zwahlen et al., 1997). Coordinates for the NMR structures of unbound TgMIC6-EGF2 and the TgMIC1-GLD–TgMIC6-EGF2 complex have been deposited at the Protein Databank under the accession codes 2K2T and 2K2S. For detailed descriptions, see the supplementary information online.

**Supplementary information** is available at EMBO reports online (http://www.emboreports.org).

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**CONFLICT OF INTEREST**

The authors declare that they have no conflict of interest.

**REFERENCES**


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3.3 Detailed insights from microarray and crystallographic studies into carbohydrate recognition by microneme protein 1 (MIC1) of *Toxoplasma gondii*.

Lectins display variable ligand specificities (Varki, 1997). Discrimination between ligands occurs by type and conformation of the sugar and by sugar modifications such as acetylation and sulphation. It may also involve the linkage to the underlying sugar chain and its composition. Additional contacts to the protein moiety of a glycoconjugate may further enhance the specificity of the interaction.

Carbohydrate microarray analyses revealed a preference of TgMIC1-MARR for 2-3 linked sialyl-oligosaccharides (Blumenschein et al, 2007). The molecular and structural details that confer this specificity are analysed in this study. Dose-response assays confirmed the preference of TgMIC1-MARR for 2-3 linked over 2-6 linked sialyl-oligosaccharides. The comparison of crystal structure complexes of TgMIC1-MARR with several oligosaccharides comprising 2-3 or 2-6 linkages identified the amino acid residues involved in contacts with the sugar and demonstrated the importance of a glutamate residue for the preference of 2-3 linked sialyl-oligosaccharides. In addition two fluorinated analogues were found to be highly potent ligands. The personal contribution to this study is restricted to a cell binding competition assay using one of the fluorinated analogues to demonstrate that this compound is able to compete for TgMIC1 binding with endogenous host cell receptors.

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Detailed insights from microarray and crystallographic studies into carbohydrate recognition by microneme protein 1 (MIC1) of *Toxoplasma gondii*

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Abstract: The intracellular protozoan *Toxoplasma gondii* is among the most widespread parasites. The broad host cell range of the parasite can be explained by carbohydrate microarray screening analyses that have demonstrated the ability of the *T. gondii* adhesive protein, TgMIC1, to bind to a wide spectrum of sialyl oligosaccharide ligands. Here, we investigate by further microarray analyses in a dose-response format the differential binding of TgMIC1 to 2-3- and 2-6-linked sialyl carbohydrates. Interestingly, two novel synthetic fluorinated analogs of 30SiaLacNAc1–4 and 30SiaLacNAc1–3 were identified as highly potent ligands. To understand the structural basis of the carbohydrate binding specificity of TgMIC1, we have determined the crystal structures of TgMIC1 micronemal adhesive repeat (MAR)-region (TgMIC1-MARR) in complex with five sialyl-N-acetyllactosamine analogs. These crystal structures have revealed a specific, water-mediated hydrogen bond network that accounts for the preferential binding of TgMIC1-MARR to arrayed 2-3-linked sialyl oligosaccharides and the high potency of the fluorinated analogs. Furthermore, we provide strong evidence for the first observation of a C—F···H—O hydrogen bond within a lectin-carbohydrate complex. Finally, detailed comparison with other oligosaccharide-protein complexes in the Protein Data Bank (PDB) reveals a new family of sialic-acid binding sites from lectins in parasites, bacteria, and viruses.

Keywords: *Toxoplasma gondii*; sialic acid; crystal structure; MIC1; microneme proteins; carbohydrate microarray

James A. Garnett and Yan Liu contributed equally to this work.

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**Introduction**

*Toxoplasma gondii* is an intracellular protozoan parasite belonging to the phylum *Apicomplexa*, which includes *Plasmodium* and *Cryptosporidium* species. *T. gondii* infection is prevalent worldwide with up to half of the human population having been chronically infected. Although usually asymptomatic, infection of immunocompromised individuals such as patients with HIV/AIDS, results in toxoplasmosis, a condition that encompasses a variety of disease states including focal central nervous system infection.1,2 In pregnant women, infection can also result in fetal and infant mortality or birth defects.3 Toxoplasma infection has also been linked to subtle forms of schizophrenia4 and other psychiatric manifestations.5

*T. gondii* has two distinct parts in its lifecycle: a sexual phase occurring in the primary hosts (domestic and wild cats) and an asexual phase where the parasite can propagate in almost any warm blooded animal. During asexual reproduction, the rapidly dividing form of the parasite, the tachyzoite, quickly establishes infection in the host through recognition and forced entry into a diverse range of cell types. Unlike the typical endocytic host-uptake pathways for viral and bacterial invasion, *T. gondii* and other apicomplexan parasites can actively penetrate host cells via a highly orchestrated process initiated by the binding to receptors on the host cell surface.

Microneme proteins (MICs) are released on the parasite surface at the time of invasion and act as major cellular adhesins,5 participating in parasite reorientation and entry of the parasite into the host cell.7–9 *T. gondii* microneme protein 1 (TgMIC1) is an important host cell binding protein that associates with two other MICs, TgMIC4 and TgMIC6.10–12 The N-terminal region from TgMIC1 possesses two tandemly arranged repeats of a novel cell-binding domain named the micronemal adhesive repeat (MAR).13,14 Preliminary carbohydrate microarray screening analyses revealed that a wide spectrum of sialylated oligosaccharide structures are recognized by the MAR-region from TgMIC1 (TgMIC1-MARR).15 These include N- and O-glycans, gangliosides, and polysialic acid sequences in which sialic acid is 2-3-linked to galactose or 2-8-linked. Crystal structures of TgMIC1 complexed with sialyl carbohydrates revealed major contacts between a conserved threonine residue and the carboxyl group of the sialic acid moiety.13 The importance of sialic acid recognition for efficient host cell invasion was corroborated by cell invasion assays, showing markedly reduced levels of parasite internalization in the presence of soluble sialic acid or using cells pretreated with neuraminidase.13

In this study, we characterize in further detail the binding of TgMIC1-MARR to five sialyl trisaccharides by microarray analyses. Interestingly, differing binding potencies to sialyl ligands are observed when they are presented as multivalent probes that simulate presentation at the host cell surface.15 We demonstrate that TgMIC1-MARR is not only able to bind a diverse array of sialyl oligosaccharides but can discriminate between different oligosaccharide linkages, which may explain that while *T. gondii* has a predilection for a variety of cell types, the parasite nevertheless exhibits markedly varied virulence in vivo in different hosts. Using new crystal structures, we also provide the atomic resolution basis for the differential recognition of 3’ and 6’SiaLacNAc1–4 (with the Galβ1–4 linkage known as Type 2 backbone16; Scheme 1) by TgMIC1-MARR, and make comparisons with the Type 1 chain isomer of 3’SiaLacNAc1–4 (designated 3’SiaLacNAc1–3). Furthermore, having observed in exploratory microarray analyses that analogs of 3’SiaLacNAc1–4 and 3’SiaLacNAc1–3 with a fluorine substitution at C-2 of galactose (Gal) elicit significantly higher binding signals than their nonfluorinated analogs,17 these compounds are also examined and we observe a C–F...H–O hydrogen bond. We compare results of TgMIC1-MARR with those of the well-characterized sialic acid recognizing plant lectin wheat germ agglutinin (WGA). Furthermore, comparison with structures of other oligosaccharide-protein complexes reveals an analogous mode of interaction in three unrelated lectins, suggesting a new family of binding motifs with diverse scaffolds.

**Results**

**Synthesis of fluorinated sialyl trisaccharides to probe the ligand tolerance of TgMIC1-MARR**

Although fluorinated enzyme inhibitors have been elegantly used to investigate sugar processing mechanisms,18 effective fluorosugar ligands for cell adhesion proteins have not been. We reasoned that, as well as potentially revealing novel features in the mode of action, such unnatural oligosaccharides are also putative adhesion inhibitors. Inhibitors with abiotic substitutions (i.e., 2′F) are potentially resistant to endogenous enzyme processing. The unnatural synthetic oligosaccharides 2′F-3’SiaLacNAc1–4 and 2′F-3’SiaLacNAc1–3 were prepared through a chemoenzymatic strategy:17 suitably protected N-acetylgalcosamine (GlcNAc) derivatives were chemically glycosylated using trichloroacetimidate methodology to introduce the 2F-Gal or Gal with appropriate connectivity (β1–3 or β1–4). Sialic acid was introduced using tolerant trans-sialidase from *T. cruzi* to create the 3′-sialyl trisaccharide variants.

**Carbohydrate microarray analyses corroborate the TgMIC1-MARR preference of 2-3-linked sialyl oligosaccharides and reveal superior binding to fluorinated analogs**

To compare closely the binding responses of TgMIC1-MARR to the immobilized 3′ and 6’SiaLacNAc1–4
probes and to the 3′SiaLacNAc₁₋₃ isomer, as well as to the fluorinated probes, 2′F-3′SiaLacNAc₁₋₄ and 2′F-3′SiaLacNAc₁₋₃ (Scheme 1 and Table I), we generated microarrays in a dose-response format [Fig. 1(a,c)]. The two nonsialylated analogs LacNAc₁₋₄ and LacNAc₁₋₃ were included as negative controls. These oligosaccharides were arrayed as oxime-linked neoglycolipids with ring-closed monosaccharide cores.¹⁹

Also included in the arrays, as controls, were six naturally occurring gangliosides (Table I). In accord with earlier microarray screening data,¹³ TgMIC₁-MARR gave significant binding signals with all of the sialyl probes tested except GD₂ and GD₃, which terminate in a 2-8-linked disialyl moiety and the two neutral disaccharides LacNAc₁₋₄ and LacNAc₁₋₃ [Fig. 1(a,b) and Table I]. As the binding curves were almost linear up to 2 fmol per spot, intensities could be compared at this point, relative to that of 3′SiaLacNAc₁₋₄ taken as 1.0 (Table I) to indicate binding potencies.

The relative binding score of 3′SiaLacNAc₁₋₃, 1.2, was comparable to that of 3′SiaLacNAc₁₋₄, whereas that of 6′SiaLacNAc₁₋₄ was considerably lower, 0.3. Strikingly, the strongest binding signals, 4.1 and 3.3, were with the fluorinated compounds 2′F-3′SiaLacNAc₁₋₄ and 2′F-3′SiaLacNAc₁₋₃, respectively; they even surpassed the signals elicited by the naturally occurring gangliosides GD₁a and GT₁b.

For comparison, we performed in parallel, microarray analysis of WGA, which is a well-characterized sialic acid-binding lectin.²⁰,²¹ As with TgMIC₁-MARR, WGA gave very similar microarray responses for the nonfluorinated sialylsugars; that is no discrimination between 3′SiaLacNAc₁₋₄ and 3′SiaLacNAc₁₋₃ [Fig. 1(b,d) and Table I], and a clear discrimination between 3′SiaLacNAc₁₋₄ and 6′SiaLacNAc₁₋₄ (1.0 and 0.1, respectively), in accord with previous observations.¹⁹,²₀ However, unlike TgMIC₁-MARR, WGA gave much lower binding signals with the two fluorinated probes, 2′F-3′SiaLacNAc₁₋₄ and 2′F-3′SiaLacNAc₁₋₃ (0.5 and 0.4, respectively), relative to their unmodified analogs.

To investigate whether the stronger binding of TgMIC₁-MARR to the fluorinated sialyl trisaccharides was specific and to compare the binding strengths of 2-3-linked and 2-6-linked sialyl oligosaccharides in the monovalent state, we performed “on-array” inhibition assays using free oligosaccharides 3′ and 6′SiaLacNAc₁₋₄. An unrelated neutral trisaccharide, maltotriose (Glc₁₋₄Glc₁₋₄Glc) was included as a negative control. We observed that 3′SiaLacNAc₁₋₄ inhibited the high avidity binding to the immobilized fluorinated trisaccharide 2′F-3′SiaLacNAc₁₋₄, indicating that binding was specific; there was 40% inhibition at 1 mg/mL and almost complete inhibition at the highest concentration tested, 3 mg/mL. Also in accord with the binding data, the 6′SiaLacNAc₁₋₄ was less active: there was no inhibition detected at 1 mg/mL; and ~70% inhibition at 3 mg/mL [Fig. 2(a)]. The binding of WGA to the immobilized 3′SiaLacNAc₁₋₄ probe was also inhibited more strongly by 3′SiaLacNAc₁₋₄ than by 6′SiaLacNAc₁₋₄ [Fig. 2(b)]. To test whether the fluorosugars were specific inhibitors of the sialic acid-dependant cell binding,¹³ a competition cell binding assay was performed with 2′F-3′SiaLacNAc₁₋₄. Indeed, effective inhibition of cell binding by TgMIC₁ was achieved in 10–500 μM range of 2′F-3′SiaLacNAc₁₋₄ [Fig. 2(c)].
Overall structures
The preference of 3’SiaLacNAc<sub>1–4</sub> over the 2-6-linked analog, and the superior binding of TgMIC1-MARR to the fluorinated sialyl trisaccharides in the microarray analyses prompted us to look into the details of the interactions at the atomic level. The crystal structures of TgMIC1-MARR in complex with either 2’F-3’0SiaLacNAc<sub>1–4</sub> or 2’F-3’0SiaLacNAc<sub>1–3</sub> were solved at 2.0 Å (Table II). Residues 1–12 and 244–246 were not observed in the crystal structures, probably due to flexibility at the termini. Overall the electron density is well resolved, although within the poorest region of the maps (the disordered loop between Q80 and N82) the atomic positions could not be determined and were either omitted from the final model (3’0SiaLacNAc<sub>1–3</sub> and 2’F-3’0SiaLacNAc<sub>1–4</sub> soaks) or modeled as polyalanine (2’F-3’0SiaLacNAc<sub>1–4</sub> soak).

Although it was possible to model all of the carbohydrate chain in the 3’SiaLacNAc<sub>1–3</sub> and 2’F-3’SiaLacNAc<sub>1–4</sub> soaks, electron density for the GlcNAc residue of 2’F-3’0SiaLacNAc<sub>1–4</sub> was of insufficient quality to permit its inclusion in the final model [Fig. 3(a–c)].

Each of the two MAR domains is formed from a distorted five strand β-barrel packed against two helices and contains two highly conserved disulphide bonds. The N-terminal MAR domain (MAR1) has an extended helix 1 and subsequent loop region that is anchored via an additional disulfide bond while the C-terminal MAR domain (MAR2) has an extra β-wing at the C-terminus, which is anchored to the main body through two additional disulfide bridges. The crystal structures of TgMIC1-MARR-3’0SiaLacNAc<sub>1–4</sub> (pdb:2Jhd), TgMIC1-MARR-6’0SiaLacNAc<sub>1–4</sub> (pdb:2Jh7) and the structures described here were superimposed onto unliganded TgMIC1-MARR (pdb:2Jh1) giving an average r.m.s.d between Cα residues of 0.18 Å. As anticipated the overall secondary structures of TgMIC1-MARR are indistinguishable from those published [Fig. 3(d)], although minor differences occur in the ligand binding site (see below) and deviations are observed within a dynamic loop region corresponding to residues L76-N82.

Carbohydrate-TgMIC1-MARR interactions
Because of the lack of global and local changes to the structure of TgMIC1-MARR upon binding of sialylated oligosaccharides, a detailed analysis of hydrogen bonding between the protein and carbohydrate was undertaken (Table III and Fig. 4) to provide explanations for the differing binding avidities and the intriguing
enhancements with fluorinated ligands observed in microarray experiments.

All direct hydrogen bond interactions between the trisaccharides and TgMIC1-MARR are between the sialic acid (NeuAc) residue and three residues in the MAR2 domain: K200 (two hydrogen bonds), H202 (two hydrogen bonds), and T204 (three hydrogen bonds). In all structures except $3'-\text{SiaLacNAc}_{1-4}$, a structured water molecule (WAT170) mediates intracarbohydrate interactions between the NeuAc ring oxygen, NeuAc glycerol chain and Gal, stabilizing the conformation of the glycerol moiety such that its two terminal hydroxyls can hydrogen bond to T204 and E206 via another water molecule (WAT145) [Fig. 4(a–d,g–j)]. Although the glycerol moiety in $3'-\text{SiaLacNAc}_{1-4}$ is rotated so that H-bonds to WAT145 cannot be made, the relative orientation of the NeuAc and Gal rings of the carbohydrate is very similar to that observed for $3'-\text{SiaLacNAc}_{1-3}$. In all the complexes of all the 2-3-linked sialyl trisaccharides [Fig. 4(a–h)], an addition water molecule (WAT129) mediates interactions between TgMIC1-MARR E206 and the Gal O6 position.

Although the GlcNAc residue at the reducing end is well resolved in the electron density maps of the $3'-\text{SiaLacNAc}_{1-3}$ and fluorine substituted structures [Fig. 3(b,c)], it makes no contacts with the MAR2 binding domain. Its position appears to be stabilized by contacts from symmetry-related proteins in the crystal; in solution this group is likely to be disordered. In the $3'-\text{SiaLacNAc}_{1-4}$, $2'F-3'-\text{SiaLacNAc}_{1-4}$ and $6'-\text{SiaLacNAc}_{1-4}$ bound structures, these additional

Figure 1. Microarray analyses of TgMIC1-MARR and WGA in a dose response format. Eight lipid-linked oligosaccharide probes, a–h, (sequences in Table I) were printed in duplicate at the indicated levels on nitrocellulose-coated glass slides with Cy3 dye included as a marker (green emission). Binding was detected with Alexa Fluor647-labeled streptavidin (red emission). Images for TgMIC1-MARR (40 μg/mL) and WGA (1 μg/mL) are in panels (a) and (b), respectively. Corresponding binding curves are in (c) and (d), respectively. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Figure 2. Comparison of the inhibitory activities of free oligosaccharides toward the binding of TgMIC1-MARR. Oligosaccharides $3'-\text{SiaLacNAc}_{1-4}$, $6'-\text{SiaLacNAc}_{1-4}$, and maltotriose (a negative control) were tested at the indicated concentrations for inhibition of binding of TgMIC1-MARR at 4 μg/mL, to immobilized $2'F-3'-\text{SiaLacNAc}_{1-4}$ probe (a), and of WGA at 0.5 μg/mL to immobilized $3'-\text{SiaLacNAc}_{1-4}$ probe (b). The results are means of two separate inhibition experiments with error bars. Cell binding competition experiments with soluble $2'F-3'-\text{SiaLacNAc}_{1-4}$ were performed using P. pastoris culture supernatants expressing TgMIC1myc. Anti-myc antibodies were used as to probe for bound TgMIC1 using Western blots. The input (I) and cell bound fraction at various concentrations of soluble $2'F-3'-\text{SiaLacNAc}_{1-4}$ are shown. Tubulin was used as a control for use of equivalent amounts of cell-material (c).
interactions are not accessible because of steric clashes between the GlcNAc and symmetry-related TgMIC1-MARR; this residue remains disordered and is not seen in the maps [Fig. 3(a)]. These observations are consistent with microarray data, in which TgMIC1-MARR binds equally well to Type 1 and Type 2 backbones.

In contrast to the lack of discrimination of Type 1 and Type 2 backbones, a consistent and reproducible preference for 3'SiaLacNAc1–4 relative to 6'SiaLacNAc1–4 is observed in the microarray analyses. The structure of 6'SiaLacNAc1–4 bound to Tg-MIC1-MARR, reveals a similar network of interactions for the NeuAc moiety to those observed in the 2-3-linked structures [Fig. 4(i)]. However, the 2-6 sialyl linkage results in Gal being flipped by about 180° which puts the ring oxygen (O5) in a position close (~2 Å) to that occupied by the Gal O2/F2 in the other oligosaccharide structures [Fig. 4]. Stabilization of the oligosaccharide conformation is still mediated through WAT170, but this water has moved closer toward Gal O6 and the hydrogen bonding to galactose is via the Gal ring oxygen (O5) instead of Gal O2/F2 as in the other crystal structures [Fig. 4].

Stabilization of the oligosaccharide conformation is still mediated through WAT170, but this water has moved closer toward Gal O6 and the hydrogen bonding to galactose is via the Gal ring oxygen (O5) instead of Gal O2/F2 as in the other crystal structures. Most striking, however, is the finding that this altered galactose orientation abrogates the WAT129-mediated interactions between TgMIC1-MARR and Gal that is observed in the structures of all the 2-3 linked SiaLacNAc1–4 analogs [Fig. 4(a–d)].

Fluorination of 3'SiaLacNAc1–3 at Gal C2 has no effect on the bound conformation of the carbohydrate moieties [Figs. 4(a–d) and 5(a)] water molecules. However, fluorination of 3'SiaLacNAc1–4 at Gal C2 has a significant effect on both carbohydrate structure [Figs. 4 and 5(b)] and hydrogen bond network. In this case, the glycerol side chain is flipped in the nonfluorinated version and WAT170 is absent. The introduction of the fluorine atom appears to stabilize the hydrogen bond formation with the bound water, WAT170 [Fig. 4(g)], with a concomitant change in conformation of interactions are not accessible because of steric clashes between the GlcNAc and symmetry-related TgMIC1-MARR; this residue remains disordered and is not seen in the maps [Fig. 3(a)]. These observations are consistent with microarray data, in which TgMIC1-MARR binds equally well to Type 1 and Type 2 backbones.

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### Table II. Data Collection and Refinement Statistics

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<td>P4₃22</td>
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Numbers in parentheses refer to the outermost resolution shell.

R<sup>sym</sup> = \[ \frac{\sum |I - \langle I \rangle|}{\sum I} \]

where \( I \) is the integrated intensity of a given reflection and \( \langle I \rangle \) is the mean intensity of multiple corresponding symmetry-related reflections.

R<sup>work</sup> = \[ \frac{\sum |F_o - |F_c||}{\sum F_o} \]

R<sup>free</sup> = R<sup>work</sup> calculated using ~10% random data excluded from the refinement.

rmssd stereochemistry is the deviation from ideal values.

Ramachandran analysis was carried out using PROCHECK.²²

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2023-02-01
Table III. Interatomic Distances Between TgMIC1-MARR and Carbohydrate Ligand

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<tr>
<th>Distance (Å)</th>
<th>3′SiaLacNAc&lt;sub&gt;1–4&lt;/sub&gt; soak</th>
<th>3′SiaLacNAc&lt;sub&gt;1–3&lt;/sub&gt; soak</th>
<th>2′F-3′SiaLacNAc&lt;sub&gt;1–4&lt;/sub&gt; soak</th>
<th>2′F-3′SiaLacNAc&lt;sub&gt;1–3&lt;/sub&gt; soak</th>
<th>6′SiaLacNAc&lt;sub&gt;1–4&lt;/sub&gt; soak</th>
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Figure 4. The occupied binding site of TgMIC1-MAR2. (a, b) 3'SiaLacNAc1–3 soak, (c, d) 2'F-3'SiaLacNAc1–3 soak, (e, f) 3'SiaLacNAc1–4 soak, (g, h) 2'F-3'SiaLacNAc1–4 soak, and (i, j) 6'SiaLacNAc1–4 soak. (a, c, e, g, i) Schematic representation of the occupied TgMIC1-MAR2 binding site with hydrogen bonds shown as red dashed lines, waters as numbered red circles and key atoms within the carbohydrate ligand are numbered (water molecules 1129, 1145, and 1170 in the 2'F-3'SiaLacNAc1–4, 3'SiaLacNAc1–3 and 2'F-3'SiaLacNAc1–3 soak structures are renamed as 129, 145, and 170, respectively, for consistency with the 3'SiaLacNAc1–4 and 6'SiaLacNAc1–4 soaks). (b, d, f, h, j) Crystal structure of the occupied TgMIC1-MAR2 binding site, with carbon atoms coloured as in Figure 3(d) and hydrogen bonds shown as red dashed lines. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]
the NeuAc glycerol side chain to the same as that observed in the other structures.

**Discussion**

*T. gondii* is uniquely adapted to infect a wide range of hosts and to invade virtually all cell types. The remarkable range of sialyl oligosaccharide sequences recognized by TgMIC1-MARR in carbohydrate microarrays accounts for this wide cellular tropism. Sialic acid containing carbohydrates are found at the termini of animal glycoconjugates. Recognition of the sialic acid moiety is often affected by specific structural variations of the monosaccharide and its linkage along the sugar chain. Although TgMIC1-MARR does not discriminate between Type 1 and Type 2 backbones, a consistently observed preference is for 2-3 over 2-6-linked sialyl oligosaccharides.

The major oligosaccharide binding activity lies within the second MAR domain (MAR2), with a major contact occurring between a threonine residue (T204) and the carboxyl group of sialic acid. An extensive database analysis of other oligosaccharide-protein complexes reveals an analogous mode of interaction in three lectins unrelated to TgMIC1, namely the *Bordetella pertussis* toxin, Staphylococcal superantigen-like (SSL) proteins, and the Rotavirus spike-associated carbohydrate binding domain25–28 (see Fig. 6). These lectins do not possess MAR domains but recognize sialyl oligosaccharides using a similar motif (see Fig. 6) that comprises a short, linear stretch of amino acids containing an essential threonine/serine residue. The structures overlay with great similarity, although the arginine is absent in TgMIC1 this role has been replaced by WAT145, it appears that within a hydrophilic environment a universal binding site exists for sialic acid. Interestingly, although the structures of these binding sequences are very similar, the motif is embedded in unrelated protein scaffolds suggesting convergence to a common binding function. Thus, our structural studies provide detailed insight into carbohydrate recognition by this new family of sialic-acid binding sites. Extending the search of the protein database (PDB) for 3’SiaLac containing motifs bound to unrelated lectins; examples include *Maackia amurensis* leukoagglutinin29 (green) (pdb:1DBN), mouse sialoadhesin30 (orange) (pdb:1QFO), Staphylococcal enterotoxin B31 (blue) (pdb:1SE3), and human Siglec-532 (yellow) (pdb:2ZG3). In these cases, the overall conformation of 3’SiaLac in these complexes vary significantly, which is likely due to the inherent flexibility in the linkage between the two sugars.

Structural comparisons between the 2-3- and 2-6-linked sialyl oligosaccharides bound to TgMIC1-MAR2...
reveal that although recognition is primarily through the terminal NeuAc residue several contributions from coordinated water molecules are important for the Gal ring orientation (see Fig. 4). WAT129 mediates the only interaction between protein (E206) and Gal O6 position in the 2-3-linked sequences examined. In contrast, in the 2-6-linked analog, the Gal O6 position is occupied by the sialic acid linkage and the Gal residue is shifted away from the sialic acid moiety by one bond, leading to the loss of the WAT129 water-coordinated carbohydrate-protein contact. The absence of this structured water molecule explains the observed binding preference for 2-3-linked sialyl oligosaccharides in our microarray analyses. Furthermore, our findings also indicate that E206 is important for determining this binding preference (see Fig. 4). Another contributing factor is the increased flexibility of the 2-6 linkage over their 2-3 counterparts, which would contribute an additional entropic penalty upon ordering in the complex.

Relative binding responses were greatly enhanced to unnatural sialyl trisaccharides that were fluorinated at the C2 position of the Gal residue. Moreover, these binding signals were greater than those with the natural glycosyl ceramides GD1a and GTb (Table I), which are among the best ligands in our previous screening microarray studies.33 The increased binding of TgMIC1-MARR to the fluorinated compounds could be fully inhibited by the natural glycan sequence 3’SiaLacNAc–1–4. Furthermore, host cell binding could be competitively inhibited with soluble fluorinated sugars indicating that the binding is specific. Such enhanced binding of fluorosugar ligand analogs to a lectin is unprecedented. Interestingly, despite recording the highest binding potencies in microarray experiments, many structural aspects of the fluorinated analogs are very similar to their hydroxyl counterparts.

In the structures of 3’SiaLacNAc–1–3 and 6’SiaLacNAc–1–4 complexes the sialic acid and galactose rings coordinate a structured water molecule, namely WAT70. WAT70 resides in a highly electronegative environment and while it has no direct contacts with the protein, it is coordinated by intracarbohydrate hydrogen bonds with NeuAc O6 (ring oxygen), NeuAc 7-OH, and Gal O2 positions. The introduction of the fluorine atom at the C2 position of the Gal molecule decreases the geometry of the hydrogen bond network. Furthermore, molecular orbital calculations and empirical observation both suggest that C—F groups are more likely to accept hydrogen bonds when in an electron rich environment.33–34 The binding site of the bound complex of TgMIC1-MARR is indeed highly electronegative, with numerous oxygen atoms participating in intraoligosaccharide and intermolecular hydrogen bonds, and this most probably contributes to the productive participation of a fluorine atom.

The affinities of individual carbohydrate-protein interactions are low, which can be problematic when probing these interactions in the monovalent state in solution. Polyvalency is a critical feature of cell surface carbohydrate recognition.34 In the carbohydrate microarray system used here, the oligosaccharides are presented as clustered lipid-linked probes, with potential for some lateral mobility, in two dimensional arrays on a chip surface.35 This might offer a suitable mimic of the arrangement of clustered oligosaccharide structures at the cell surface, and allow for polyvalent binding, resulting in a much more realistic binding response. TgMIC1 is anchored on the parasite surface.
in abundant multimeric assemblies in which at least two MIC1 molecules are present per complex. The subtle affinity and specificity differences revealed by our structural data are most probably amplified in natural host cell interactions and effectively recapitulated in the microarray experiments.

The high avidity of binding of TgMIC1 to clustered natural ligands such as gangliosides that are abundant on neuronal cells is likely a factor for the tropism of *T. gondii* in the human brain. Furthermore, potent and rapid binding to a variety of sialylated receptors may play a role in a primary interaction, perhaps to sialic acid on the gut epithelial cell wall thereby preventing excretion of the parasite from the intestine after ingestion. Although TgMIC1 binds well to both clustered 2-3 and 2-6-linked sialyl sugars, the differential binding affinity may also contribute to the diverse spectrum of *T. gondii* virulence observed in mammalian hosts.

Our findings on the preferential binding of TgMIC1 to synthetic carbohydrates such as the fluorinated compounds could potentially be exploited in the design of therapeutic receptor analogs. Such treatments would be particularly important for immunocompromised patients who are at especially high risk. Our observations on a distinctive sialic acid-binding motif containing the essential threonine/serine residue shared between TgMIC1-MARR and three unrelated adhesive proteins provides the basis for the design of novel synthetic lectins able to discriminate various sialyl oligosaccharides.

### Methods

#### Materials

Oligosaccharides 3’ and 6’SiaLacNAc<sub>1–4</sub>, were from Dextra (Reading, UK). LacNAc<sub>1–3</sub>, 3’SiaLacNAc<sub>1–3</sub>, and the fluorinated analogs of 3’SiaLacNAc<sub>1–4</sub> and 3’SiaLacNAc<sub>1–3</sub> designated 2’F-3’SiaLacNAc<sub>1–4</sub> and 2’F-3’SiaLacNAc<sub>1–3</sub>, respectively were chemically synthesized. LacNAc and maltotriose were from Sigma. The natural glycolipids (Table I): hematoside and sialyl paragloboside (Sial pg) were gifts of Professor Peter Hanfland (University of Bonn); GD1a was from Sigma; GD2 and GT1b were from HyTest (Turku, Finland); GD3 was from BioCarb (Lund, Sweden). Mouse monoclonal anti-poly-histidine and biotinylated goat anti-mouse IgG antibodies were from Sigma. Biotinylated WGA was from Vector Laboratories (Peterborough, UK).

#### Expression of recombinant soluble TgMIC1-MARR

His-tagged TgMIC1-MARR (residues 17–262) was expressed and purified as described.

#### Carbohydrate microarray binding assays

The sialyltrisaccharides (Scheme 1) and the two LacNac analogs were converted into oxime-linked neoglycolipids for arraying. These neoglycolipid probes, together with natural glycolipids, were robotically printed onto 16-pad nitrocellulose glass slides, the layout of each pad being in a dose-response format [Fig. 1(a,c)]. Microarray binding analyses with the recombinant His-tagged TgMIC1-MARR were performed essentially as described. In brief, the His-tagged TgMIC1-MARR was precomplexed with mouse monoclonal anti-poly-histidine and biotinylated anti-mouse IgG antibodies in a ratio of 1:1.25:1.25 (by weight). A control experiment was carried out in the absence of TgMIC1-MARR and no binding signal was detected. The binding analysis of WGA was performed as described.

#### On-array inhibition of TgMIC1-MARR and WGA binding using free oligosaccharides as inhibitors

Binding of TgMIC1-MARR at 4 μg/mL and WGA at 0.5 μg/mL were carried out in the presence of free oligosaccharides 3’SiaLacNAc<sub>1–4</sub> and 6’SiaLacNAc<sub>1–4</sub>, as well as maltotriose (a negative control). The three
oligosaccharides were preincubated, at different concentrations as indicated (see Fig. 2), to the TgMIC1-MARR (precomplexed) or WGA before overlaying on the microarray slides. The percentage of inhibition of binding in the presence of inhibitors was determined as follows using the fluorescence intensity observed with arrayed neoglycolipid probe of 2’F-3’SiaLacNAc1–4 printed at 5 fmol/spot in the microarray: % Inhibition = [(means of duplicate spots with maltotriose – means of duplicate spots with 3’ or 6’SiaLacNAc1–3)/means of duplicate spots with maltotriose] × 100%.

**Competition cell binding assay**

These were performed as described previously using 2’F-3’SiaLacNAc1–4 as the soluble ligand.

**Crystallization and data collection**

TgMIC1-MARR was crystallized as described. Crystals were soaked overnight in 3.5 mM ammonium acetate, 100 mM bis-tris propane pH 7.0, and either 3.0 mM of 2’F-3’SiaLacNAc1–4, 3’SiaLacNAc1–3 or 2’F-3’SiaLacNAc1–3. Before data collection, crystals were soaked in cryoprotectant and then frozen immediately. Data were collected at 100 K on beamline ID14-1 at the European Synchrotron Radiation Facility (ESRF).

**Structure solution and refinement**

All data were processed with MOSFLM and scaled using SCALA. Phases were obtained by molecular replacement using PHASER with TgMIC1-MARR (pdb:2jh1) as the search model. Approximately 10% of the data were used to calculate Rfree. Refinement was carried out with REFMAC5 and model building was performed with Coot. During the final stages of refinement TgMIC1-MARR (chain A) was submitted to the TLS motion determination server. The modified PDB and TLS input files (with a single TLS parameter describing the chain) were used with REFMAC5 for a restrained and TLS B-factor refinement. The protein structures were validated with PROCHECK and the carbohydrate structures validated using pdb-care. Data processing and final refinement statistics are given in Table I. Superimposition of molecules was performed with Lsgkab and intermolecular distances were measured using CONTACTS. The coordinates and structure factors for the TgMIC1-MARR 2’F-3’SiaLacNAc1–4, 3’SiaLacNAc1–3 and 2’F-3’SiaLacNAc1–3 soaks have been deposited with the PDB under ID code 3f53, 3f5a, and 3f5e, respectively.

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3.4 A novel protein family containing MAR domains act as sialic acid lectins during Apicomplexa invasion

The results presented in section 3.1. demonstrated that sialic acid is a major determinant of host cell invasion by *T. gondii*, but the parasite ligands important for sialic acid-dependent invasion remained undetermined. Since we showed that TgMIC1 specifically recognizes sialylated oligosaccharides on the host cell surface, this protein is an obvious candidate. A previously generated TgMIC1 knock-out strain (*mic1ko*) showed a 50% reduction in invasion efficiency compared to the wild-type strain (Cerede et al, 2005). In this strain the TgMIC1-4-6 complex is disrupted, ablating transport of TgMIC4 and TgMIC6 to the micronemes and instead resulting in their retention in the early secretory pathway (Reiss et al, 2001). In consequence, the contribution of the individual components to host cell invasion remained unresolved. In this study we have addressed this question and investigated on further candidate parasite proteins involved in sialic acid-dependent host cell invasion. This led us to the identification of a novel protein family defined by the presence of the MAR domain and the characterization of TgMIC13 and NcMIC1, the TgMIC1 homologue in *N. caninum*. Carbohydrate microarray analyses and structural modeling were employed for detailed analysis of binding specificities of TgMIC1, TgMIC13 and NcMIC1. These experiments were performed by the groups of Prof. Stephen Matthews and Prof. Ten Feizi (both from Imperial College, London). More details on the individual contributions of the authors can be found in the acknowledgements of the article.

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A novel protein family containing MAR domains act as sialic acid lectins during Apicomplexa invasion

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Abstract

Numerous intracellular pathogens exploit cell surface glycoconjugates for host cell recognition and entry. Unlike bacteria and viruses, *Toxoplasma gondii* and other apicomplexans actively invade host cells. Micronemal adhesins (MICs) play crucial roles in this process. We recently described a new structural module in *T. gondii* MIC1 (TgMIC1) termed Microneme Adhesive Repeat (MAR), which recognizes sialyl-oligosaccharides. Here, we establish TgMIC1 as one important player in sialic acid-dependent invasion and provide evidence that another sialic acid-binding lectin is involved. This led us to identify a family of MAR containing proteins in *T. gondii* and to characterize a novel sialic acid-binding lectin, TgMIC13. Binding characteristics of TgMIC13 were compared to TgMIC1 and its homologue *Neospora caninum* MIC1 by carbohydrate microarray analysis and molecular modelling. While all three share a preference for α2-3-linked sialyl-oligosaccharides, binding specificities are distinct, and this might have implications for tissue tropism. Conservation of the MAR family among coccidians suggests that all these enteroparasites exploit sialylated glycoconjugates on the host cell surface as major determinants for a broad host cell range and enteric invasion.
**Introduction**

Sialic acids occur abundantly in glycoproteins and glycolipids on the cell surface, and are exploited by many viruses and bacteria for attachment and host cell entry. Recognition of carbohydrates and in particular sialylated glycoconjugates is important also for host cell invasion by the Apicomplexa (Blumenschein et al, 2007; Persson et al, 2008; Sim et al, 1990; Takabatake et al, 2007), a phylum which comprises several thousand species of obligate intracellular parasites. Among them the *Plasmodium* spp. are responsible for malaria, *Eimeria* spp. for coccidiosis in poultry, *Neospora* spp. for neosporosis in cattle, and *Toxoplasma* is the causative agent of toxoplasmosis in warm blooded animals and humans.

The host range and cell type specificity varies widely across the phylum. In contrast to *Plasmodium falciparum* merozoites that exclusively invade erythrocytes of humans and great apes (Martin et al, 2005), *T. gondii* rapidly establishes infection in the host by the fast-replicating tachyzoite form which disseminates into deep tissues and invades an extremely broad range of cell types in humans and virtually all warm blooded animals (Montoya & Liesenfeld, 2004). The molecular basis for this broad host cell recognition is not well understood.

Many intracellular pathogens have evolved to manipulate the phagocytic pathways of host cells during invasion. This contrasts with invasion by the apicomplexans, which express their own machinery for active host cell entry. Invasion is a multistep process requiring the tightly regulated discharge of parasite organelles called micronemes and rhoptries (Carruthers & Sibley, 1997). Micronemes release adhesins (MICs) onto the parasite surface, which form multi-protein complexes with non-overlapping roles in mediating motility, host cell attachment, secretion of rhoptry organelles and cell penetration (Soldati-Favre, 2008). After attachment and reorientation of the parasite, invasion induces the formation of a non-fusogenic parasitophorous vacuole derived in large part from host cell plasma membrane (Carruthers & Boothroyd, 2007). MICs across the phylum Apicomplexa share a limited
number of adhesive domains arranged in various combinations and numbers (Carruthers & Tomley, 2008). These domains are implicated in host cell recognition and attachment and are believed to contribute to host cell type specificity and hence disease pathology.

_T. gondii_ microneme protein 1 (TgMIC1) forms a complex with TgMIC4 and TgMIC6 (Brecht et al, 2001; Reiss et al, 2001) and binds to sialylated glycoconjugates on the host cell surface (Blumenschein et al, 2007). Previous studies based on gene disruption have established a critical role for the complex in host cell invasion in tissue culture and its contribution to virulence _in vivo_ (Cerede et al, 2005). The N-terminal region of TgMIC1 interacts with TgMIC4, a protein comprising six ‘apple’ domains that has been shown to bind to host cells in the presence of TgMIC1 (Brecht et al, 2001). TgMIC6 contains three EGF-like domains and is a type I membrane protein, which serves as an escorter and anchors the TgMIC1-MIC4-MIC6 (TgMIC1-4-6) complex to the parasite surface during invasion (Reiss et al, 2001). The first EGF-like domain (TgMIC6-EGF1) is cleaved off during secretory transport of the complex, probably in a post Golgi compartment (Meissner et al, 2002). Each of the remaining two EGF-like domains is able to recruit one molecule of TgMIC1 via interaction with its C-terminal galectin-like domain (Reiss et al, 2001; Saouros et al, 2005; Sawmynaden et al, 2008) (for a schematic of the wild-type complex see **Fig. 1A**). Correct trafficking of the complex to the micronemes depends not only on a sorting determinant in the C-terminal tail of TgMIC6, but also on the interaction between the galectin-like domain of TgMIC1 with the third membrane-proximal EGF-like domain of TgMIC6 (TgMIC6-EGF3). This interaction is crucial for transport of the entire complex through the early secretory pathway as it assists proper folding of TgMIC6-EGF3, providing a quality control check point (Huynh et al, 2004; Reiss et al, 2001; Saouros et al, 2005).

Several studies have shown that recognition of carbohydrate structures on the host cell surface is critical for efficient invasion by _T. gondii_ (Carruthers et al, 2000; Monteiro et al, 1998; Ortega-Barria & Boothroyd, 1999). We have recently demonstrated that the N-terminal
region of TgMIC1 contains two copies of a novel domain Microneme Adhesive Repeat (MAR) and that this region termed TgMIC1-MARR binds specifically to sialylated oligosaccharides as shown by cell binding assays and carbohydrate microarray analyses (Blumenschein et al, 2007). Also, we observed a 90% reduction of invasion efficiency when N-acetylneuraminic acid (NANA) was used as a competitor or when host cells were treated with neuraminidase (Blumenschein et al, 2007). This has suggested that sialic acid is a major determinant of host cell invasion by *T. gondii* and that the effect observed in the invasion assays may be attributed, at least in part, to the inhibition of the interaction between TgMIC1 and its host cell receptor(s).

Here, we compare the invasion efficiency of several *T. gondii* knockout strains and complemented mutants and demonstrate that this interaction is indeed important for efficient host cell invasion. However, we also show that the TgMIC1-4-6 complex is not the only molecular player involved in sialic acid-dependent invasion by *T. gondii* tachyzoites. This has led us to identify a complete family of MAR domain containing proteins (MCPs) in *T. gondii* and related apicomplexan parasites. Among these we characterize TgMIC13, and we compare the binding specificities of recombinant TgMIC13, TgMIC1 and its homologue from the closely related organism *Neospora caninum*, NcMIC1, by carbohydrate microarray and cell binding assays. Our results reveal that the MAR domain is unique to and conserved among coccidian parasites, and acts as an important determinant in host cell recognition by this subclass of Apicomplexans through selective binding to sialylated glycoconjugates.
Results

1. TgMIC1 is an important player in sialic acid-dependent host cell invasion by T. gondii

A previously generated TgMIC1 knock-out strain \( (\text{mic1ko}) \) showed a 50% reduction in invasion efficiency compared to the wild-type strain (Cerede et al, 2005). In this strain the TgMIC1-4-6 complex is disrupted, ablating transport of TgMIC4 and TgMIC6 to the micronemes and instead resulting in their retention in the early secretory pathway (Reiss et al, 2001). In consequence, the contribution of the individual components to host cell invasion remained unresolved (for a schematic of the complex see Fig. 1A). To address this question we complemented the \( \text{mic1ko} \) strain with three different TgMIC1-mutant constructs, then examined whether expression of these proteins is able to improve or restore invasion efficiency. A first parasite mutant line named \( \text{mic1ko/mycMIC1-GLD} \) expresses the MIC1 carboxy-terminal galectin-like domain (TgMIC1-GLD) on the \( \text{mic1ko} \) background. A second parasite line called \( \text{mic1ko/MIC1TTAAmyc} \) was generated using a construct coding for full length TgMIC1 carrying threonine to alanine substitutions at two positions (126 and 220) that were previously shown to be critical for the host cell binding activity of the TgMIC1-MAR region (TgMIC1-MARR) (Blumenschein et al, 2007). A third complemented line called \( \text{mic1ko/MIC1T220Amyc} \) expresses MIC1 containing just a single T220A substitution that also abolishes the host cell binding activity of the protein (Blumenschein et al, 2007). As a control the \( \text{mic1ko} \) strain was complemented with full length wild-type TgMIC1 carrying a myc tag epitope at the carboxy-terminus (parasite line \( \text{mic1ko/MIC1myc} \)). Western blots revealed comparable levels of expression for the respective mutant proteins (Fig. 1B). In addition, the previously generated \( \text{mic4ko} \) line (Reiss et al, 2001) was included in the analysis. Figure 1F shows a comparison of invasion efficiency of the parental wild-type and the \( \text{mic1ko} \) lines and
confirms the 50% reduced invasion phenotype previously reported for the *mic1ko* (Cerede et al, 2005).

Given that TgMIC4 and TgMIC6 are retained in the early secretory pathway in the *mic1ko* strain, assessment of the sub-cellular localisation of the TgMIC1-mutant proteins, as well as of TgMIC4 and TgMIC6, in the complemented *mic1ko* parasite lines was important for interpretation of the invasion assay results. Expression of TgMIC1myc in the *mic1ko* strain rescued the targeting of TgMIC4 and TgMIC6 to the micronemes [(Reiss et al, 2001); and suppl. Fig.1]. Invasion efficiency in this strain was restored to a level slightly higher than the parental wild-type line (Fig. 1F), confirming that a complex composed of endogenous TgMIC4 and TgMIC6 together with the epitope-tagged TgMIC1myc is functional in invasion. In agreement with previous studies (Saouros et al, 2005) expression of TgMIC1-GLD in the *mic1ko* strain brought TgMIC6 to the micronemes but TgMIC4 remained in the ER (Fig. 1C). In the invasion assay, when compared to *mic1ko*, *mic1ko/mycMIC1-GLD* showed no rescue of phenotype (Fig. 1F), demonstrating that the TgMIC6-TgMIC1-GLD mutant complex does not contribute to invasion, likely due to the absence of adhesive domains.

In the *mic1ko/MIC1T220A*myc line, TgMIC1T220A targeted to the micronemes and restored proper trafficking of TgMIC4 and TgMIC6 (Fig. 1D). This mutant invaded significantly better than *mic1ko/mycMIC1-GLD* (Fig. 1F) despite the fact that the mutation in TgMIC1T220A abrogates the adhesive properties of the protein (Blumenschein et al, 2007). This suggested that the invasion enhancement observed upon expression of this protein might be solely a result of its capacity to recruit TgMIC4 to the membrane-bound complex and/or residual weak binding of the first TgMIC1 MAR domain. Invasion efficiency of this line was similar to the wild-type but lower than the control line *mic1ko/MIC1myc*, underlining the importance of the adhesive properties of TgMIC1. In the *mic1ko/MIC1TTAA*myc line, TgMIC1TTAA was found mainly in the early secretory pathway probably due to incorrect
folding of this mutant protein (Fig. 1E), precluding any confirmation of the above results with the single T220A mutant. No improvement of invasion efficiency compared to the mic1ko was found for this line, as expected (Fig. 1F).

In the mic4ko strain, the partial TgMIC1-6 complex was delivered to the micronemes (Reiss et al, 2001). This strain invaded more efficiently than mic1ko and mic1ko/mycMIC1-GLD (t-tests: p<0.05 respectively), confirming the critical role of TgMIC1-MARR in invasion (Fig. 1F). In addition the mic4ko strain did not invade as well as the wild-type (t-test: p<0.05), suggesting a role for TgMIC4 in invasion in agreement with our observations with the mic1ko/MIC1T220A myc line, or alternatively that the presence of MIC4 impacts on the proper function of the MAR domains.

Collectively, the characterization of these mutants suggests a cooperative function for TgMIC1-MARR and TgMIC4 in receptor binding and confirms the absence of any adhesive function for TgMIC6 and TgMIC1-GLD. Furthermore, these experiments establish that the binding activity of TgMIC1-MARR to sialylated structures recapitulates TgMIC1 function and its contribution to efficient invasion.

2. T. gondii possesses more than one sialic acid binding factor involved in host cell invasion

To address the question of whether other parasite lectins bind to host sialylated glycoconjugates during invasion, we tested the effect of free NANA on invasion by the mic1ko strain. Interestingly, this assay showed that host cell invasion by the mic1ko strain was still considerably impaired in the presence of free NANA (Fig 2A), indicating that TgMIC1 is not the only sialic acid-binding parasite lectin contributing to invasion by T. gondii tachyzoites. This result was confirmed by performing an invasion assay with neuraminidase-
treated host cells which resulted in a substantial reduction of invasion by the \textit{mic1ko} strain (Fig 2B).

3. Identification of a novel family of MAR containing proteins (MCPs) in Coccidia

In the light of the observation that host cell invasion by \textit{mic1ko} parasites is still sensitive to inhibition by free NANA, we hypothesized that \textit{T. gondii} has at least one additional sialic acid-binding parasite lectin. A survey of the \textit{T. gondii} genome sequence revealed that it encodes a family of four MAR-domain containing proteins (MCPs), herein named TgMIC1 (80.m00012), TgMCP2 (55.m04865), TgMCP3 (25.m00212) and TgMCP4 (25.m01822) (see Fig. 3A and suppl. Fig. 2). The presence in all cases of a predicted amino-terminal signal peptide suggests that these proteins are delivered to the secretory pathway. In contrast to TgMIC1, which comprises two MAR domains (TgMIC1-MARR) followed by a galectin-like domain (TgMIC1-GLD), the other three sequences contain four consecutive MAR domains but lack a galectin-like domain. In addition, TgMCP4 possesses a novel N-terminal repeat region (16 repeats, 17 to 22 amino acids per repeat), with the number of repeats varying between different strains of \textit{T. gondii}. Examination of the EST-data set indicate that all these MCP genes are transcribed both in tachyzoite and bradyzoite stages and analysis of available proteomics data (Xia et al, 2008) confirmed the expression of TgMCP2 in tachyzoites.

Our earlier studies have established that TgMIC1-MARR has the potential to bind to two molecules of sialic acid through its two binding sites, one in each MAR domain, characterized by the presence of conserved His and Thr residues. In both binding sites the threonine residue (T126 and T220 respectively) makes principle contacts to the sialic acid moiety (Blumenschein et al, 2007). Sequence comparison reveals that these important threonine residues are not present in either TgMCP3 or TgMCP4, but are conserved in three
out of the four MAR domains in TgMCP2 (suppl. Fig. 2). Consequently, we hypothesized that TgMCP2 may be another sialic acid binding parasite lectin.

An examination of related apicomplexan parasites allowed identification of a complete set of homologues in *N. caninum* (Table I and suppl. Fig. 2). NcMIC1, the homologue of TgMIC1 has been previously described (Keller et al, 2002), whereas TgMCP2 is highly similar to NC_LIV_092400 (73% id. and 86% sim.), and genes coding for proteins highly similar to TgMCP3 (67% id. and 75% sim.) and TgMCP4 (73% id. and 80% sim. in the MAR region) are present on *N. caninum* chromosome 1b, contig 996 (suppl Fig. 3 and 4 show full amino acid sequence alignments). Like TgMCP4, NcMCP4 displays an N-terminal region with short repeats, but comprising only 8 units. Interestingly, the His and Thr signature residues of the MAR sialic acid binding site are conserved when comparing the *N. caninum* and *T. gondii* homologues, suggesting that these could be functionally equivalent and hence orthologues (suppl. Fig. 2). This view is supported by a phylogenetic analysis comparing the first two predicted MAR domains of each member of the family (Fig. 3B). In this analysis the putative orthologues are indeed most closely related. TgMCP3, TgMCP4, NcMCP3 and NcMCP4 lacking the critical Thr residues form a separate cluster, consistent with an expected functional divergence.

Unfortunately, there was insufficient genomic data available to establish whether a similar gene family exists in *Sarcocystis* spp. but EST-data (SnEST4a79g11.y1, SnEST4a34d02.y1, SnESTbab01e09.y1, SnEST4a69h07.y1 and SnESTbab30h02.y1) indicates the presence of several MAR-containing proteins, one of which (termed SnMCP5) has a homologue in *N. caninum* (NC_LIV_144780 on chr.12, named NcMCP5 hereafter, see Fig. 3A and suppl. Fig. 2 and 4). Despite a high level of synteny between the two parasites, the gene encoding NcMCP5 is absent at the corresponding locus in *T. gondii*. Two additional loci coding for putative MCPs were found in the *N. caninum* genome (NC_LIV_131070 and on contig 1031, named NcMCP6 and NcMCP7 respectively) both coding for proteins
composed of a signal peptide and two MAR domains (Fig. 3A and suppl. Fig. 2 and 5). In *Eimeria tenella*, the previously described EtMIC3 contains 7 MAR domains, in which repeats three, four and five are identical (Labbe et al, 2005). EtMCP2, lying downstream of EtMIC3 on contig 29262, was annotated *in silico* by comparison with EST data from sporulated oocyst and sporozoite stages. EtMCP2 is composed of a signal peptide and a single MAR domain (Fig. 3A and suppl. Fig. 2 and 5). In addition, this parasite possesses a number of hypothetical MCPs (two gene models respectively on contigs 29652 and 14843). EST data supports expression of at least one of them in first generation merozoites. Our BLAST searches failed to identify any MCPs in *Plasmodium, Theileria* or *Cryptosporidium*. Accession numbers and proposed orthologous relationships for all proteins are depicted in Table I.

A comparison of MAR domains from *Toxoplasma, Neospora* and *Sarcocystis* highlights differences in disulphide bond patterns between two tandemly arranged MAR domains in a given MCP (suppl. Fig. 2). As in the TgMIC1-MARR prototype, the beginning of the second domain (referred to as type II) lacks a stretch of amino acids containing two cysteine residues shown to participate in the formation of a disulphide bond in the TgMIC1-MARR crystal structure, but possesses an additional C-terminal extension (with the notable exception of the second MAR domain in TgMCP2 and NcMCP2) that adopts a beta-hairpin or ‘beta-finger’ (Blumenschein et al, 2007). Curiously, in this regard all the MAR domains in EtMIC3 and in all other predicted MCPs in *Eimeria* resemble the first of the two tandemly arranged MAR domains, referred to as type I (Fig. 3A and suppl. Fig. 2). This suggests that these proteins have evolved differently in *Eimeria* compared to the MCPs found in *Toxoplasma, Neospora* and *Sarcocystis*. Therefore, despite the fact that some of the MCPs in *Eimeria* display the binding site His-Thr or a similar motif, these proteins might possess different properties and functions.
4. TgMIC13, a novel sialic acid binding lectin

A parasite line expressing a C-terminal epitope-tagged copy of TgMCP2 was generated. TgMCP2ty was co-localised with TgMIC4 to the micronemes by immunofluorescence assay (IFA) and was named TgMIC13 accordingly. IFA on wild-type parasites using antibodies raised against recombinant TgMIC13 expressed in *Pichia pastoris* confirmed localization to the micronemes, although only partial co-localization with TgMIC3 was observed (Fig. 4A). Analysis of lysates from wild type and wt/TgMIC13ty parasites by Western blot indicated that TgMIC13 migrates on SDS-PAGE as a single protein species with an apparent molecular mass somewhat higher than the predicted (51.5kDa) (Fig. 4B). TgMIC13ty migrates slightly faster than endogenous TgMIC13. The solubility profile of TgMIC13 is identical to TgMIC4 and is consistent with it being a soluble protein within the parasite organelles (Fig. 4C, upper panel). To assess whether TgMIC13 displays cell binding activity, excreted-secreted antigen fraction (ESA) prepared from wt/TgMIC13ty parasites was tested in a cell binding assay; the protein was found to bind to the cell surface (Fig. 4C, lower panel). Furthermore, transient expression of TgMIC13ty in *mic1ko* and *mic6ko* recipient strains indicated that TgMIC13 traffics to the micronemes independently of the TgMIC1-4-6 complex and does not associate with its components (Fig. 4D). These results establish that expression of TgMIC13 cannot functionally rescue the *mic1ko*, strongly suggesting that TgMIC13 belongs to a distinct complex.

The cell binding activity of TgMIC13 was reproduced with recombinant protein expressed in *P. pastoris* (Fig. 5A). Similar to TgMIC1, TgMIC13 binding was found to be abolished by pre-treatment of cells with neuraminidase (Fig. 5B). Binding of TgMIC13 to host cell receptor(s) could be competed out with free NANA, but was not affected by increasing concentrations of a control acidic monosaccharide, glucuronic acid (Fig. 5C).
However, the binding properties of TgMIC13 differed from TgMIC1 in the following ways:
(1) at equivalent concentrations, more TgMIC13 appeared to bind to HFFs (Fig. 5A); (2) compared to TgMIC1, higher concentrations of NANA were required to fully inhibit receptor binding of TgMIC13 (10 mM for TgMIC1 (Blumenschein et al, 2007) versus 50 mM for TgMIC13) (Fig. 5C); (3) only extensive treatment of cells with neuraminidase completely abolished the binding of TgMIC13 (data not shown); and (4) TgMIC1 did not bind to CHO lec2 cells that display strongly reduced surface expression of sialic acid (Deutscher et al, 1984), whereas TgMIC13 can weakly bind to them (Fig. 5D). No other differences were observed in cell binding assays with CHO mutants deficient in glycosaminoglycan synthesis (CHO-pgsA, CHO-pgsB) or with CHO cells lacking two glycosyltransferase activities (CHO-pgsD) (Esko et al, 1988) (Fig. 5D). Taken together these observations suggested that TgMIC1 and TgMIC13 have different sialic acid binding properties and may bind to different sialylated receptors.

In order to assess precisely the role of TgMIC13 in invasion, we repeatedly tried to knock-out the gene in wild-type (RH strain) and in mic1ko parasites without success. In addition, repeated knock-out attempts failed in a delta-ku80 strain efficiently amenable to gene disruption by dramatically enhanced frequency of homologous recombination (Fox et al, 2009; Huynh & Carruthers, 2009).

5. Binding properties of other T. gondii proteins of the MAR domain family

Given that the MAR domains on TgMIC1 and TgMIC13 are involved in carbohydrate recognition, we postulated that TgMCP3 and TgMCP4 could belong to a distinct functional class of lectins. In cell binding assays using ESA prepared from transgenic parasites expressing TgMCP3ty and TgMCP4ty, neither of the two epitope-tagged proteins showed
detectable binding activity (not shown). Full-length TgMCP3 as well as the MAR region of TgMCP4 (TgMCP4-MARR) encompassing all four MAR domains without the N-terminal repeat region were expressed in both *P. pastoris* and *E. coli*. No binding to HFFs could be observed with the recombinant proteins in cell-binding assays (*Fig. 5A* and data not shown). Furthermore, no binding was detected for recombinant TgMCP3 expressed and purified from *E. coli* in carbohydrate microarray analysis using neutral and sialylated probes (data not shown). While strong binding was observed for TgMIC13 expressed in *P. pastoris*, the same protein expressed in *E. coli* showed only weak binding activity reflecting the limitations of this expression system when large proteins containing numerous disulphide bonds are produced. Considering this limitation, we cannot exclude mis-folding of bacterial recombinant TgMCP3 and TgMCP4, although the lack of binding to sialic acid containing moieties would be consistent with the absence of the critical Thr residues in the MAR domains of TgMCP3 and TgMCP4 (suppl. *Fig. 2*). Homology modelling and sequence alignments suggest that while TgMCP3 and TgMCP4 adopt the MAR fold, they present a largely hydrophobic surface in the equivalent position to the hydrophilic carbohydrate binding site in TgMIC1 and therefore would not be expected to bind sugars (data not shown).

6. Carbohydrate microarray analysis reveals differing binding characteristics for TgMIC13, TgMIC1 and NcMIC1

To examine in detail the binding specificities of TgMIC13, we performed cell-independent carbohydrate microarray studies. Preliminary screening analyses with more than 300 lipid-linked oligosaccharide probes (Blumenschein et al, 2007; Feizi & Chai, 2004) showed that recombinant TgMIC13 expressed in *P. pastoris* bound exclusively to sialylated
oligosaccharide probes (data not shown). This is in accord with results of the cell binding assays, and shows that sialic acid is a requirement for TgMIC13 binding.

The fine binding specificity of TgMIC13 was compared with that of TgMIC1 and NcMIC1 using an array of 88 oligosaccharide probes. These comprised 82 diverse sialylated oligosaccharide probes with different sialyl linkages and backbone sequences (Fig. 6; suppl. Table I); 6 neutral oligosaccharide probes served as negative controls. In these experiments we used full-length TgMIC13 expressed in P. pastoris, as well as TgMIC1-MARR (aa 17-262) and NcMIC1-MARR (aa 17-259), both expressed in E. coli. TgMIC1 lacking its C-terminal galectin-like domain is known to reflect the binding specificities of the full-length protein (Blumenschein et al, 2007; Sawmynaden et al, 2008) and we assumed that this is as well the case for its homologue NcMIC1. The three proteins bound to various sialylated, but not to neutral oligosaccharides in the microarrays. Stronger binding was observed to $\alpha$2-3-linked sialyl probes compared with $\alpha$2-6 linked sialyl probes. This is in agreement with a recent study revealing the importance of a glutamic acid residue (E222) in TgMIC1 for this preference, which is conserved in TgMIC13 and NcMIC1 (suppl. Fig. 2) (Garnett et al, 2009).

Interestingly, for each protein a distinct binding profile was observed. Among the broad range of sialylated probes bound by TgMIC1-MARR, the preferred ligands are $\alpha$2-3-linked sialyl-$N$-acetyllactosamine (3′SiaLacNAc) sequences with or without fucosylation/sulphation on the $N$-acetylglucosamine residue (highlighted probes in Fig. 6A). This is in overall agreement with previous findings (Blumenschein et al, 2007). Although the oligosaccharide sequences of probes 18, 20, 21 and 22 are short tri- or tetrasaccharides, their recognition demonstrates the effective presentation of these as oxime-linked neoglycolipids on the array surface (Liu et al, 2007).

TgMIC13 bound to a more restricted spectrum of probes compared to TgMIC1-MARR (Fig. 6B). Several 3′SiaLacNAc-based probes were recognized, but there was little or
no binding to sialyl sequences having long chain polyLacNAc or N-glycan backbones (probes 38-41), and to polysialyl sequences (probes 76-85). Strikingly, the strongest binding of TgMIC13 was to a disialyllactose probe having α2-9 linkage between the two sialic acid residues (probe 88). The binding signal for this probe was extremely high (saturated) at the protein concentration tested. In addition, TgMIC13 bound to an uncommon 4-O-acetylated 3’sialyllactose (probe 16) which was not recognized by TgMIC1. It is interesting that the intensity of binding to probe 16 was stronger than that to the non-O-acetylated analogue (probe 12). For NcMIC1-MARR, there was strikingly high binding to two sialyl Le^a-related probes (33 and 37, Fig. 6C), which have in common a sulphate group at the 6 position of the galactose residue. In contrast, if the sulphation is on the N-acetylglucosamine residue as in probe 35, binding of NcMIC1-MARR was much weaker. NcMIC1-MARR gave stronger binding signals than TgMIC1 and MIC13 with polysialyl sequences.

7. Molecular modelling corroborates the findings of microarray analyses

To better understand the basis of the binding preference revealed by the microarray studies, we used the optimised sequence alignment to build homology models for the TgMIC13 MAR domains, based on the crystal structure of TgMIC1-MARR (Bordoli et al, 2009). While the first three MAR domains of TgMIC13 possess the sialic acid-binding signature, we have assumed that the type II domains (MAR2) make the dominant contribution to sialic acid recognition as this was observed for TgMIC1-MARR (Blumenschein 2007). Strikingly, the hydroxyl group on C4 of the sialic acid ring packs against the two large side chains of K216 and F169 from loops β1-β2 and β4-β5 of TgMIC1-MAR2 and would hinder any substantial modification of the sugar at this position i.e. 4-O-acetylation (Fig. 7A). In the equivalent MAR domain from TgMIC13 these bulky side-chains are replaced by smaller Ser and Thr residues, respectively, which creates space to accommodate the additional acetyl group and provide potential hydrogen-bonding partners.
In 2-9-linked disialyl oligosaccharides the two sialyl moieties are separated by three additional bonds compared to the separation between sialic acid and galactose in 3’SiaLacNAc. Assuming a similar mode of recognition for the outermost sialic acid moiety, the longer linkage would shift the second sialic acid to a position directly over the end of helix II (Fig. 7B). In TgMIC1 three consecutive Glu residues (E221-223) cap this helix, which might induce electrostatic repulsion of the negatively charged carboxylate group and hinder any preference for 2-9 disialyl sugars. In TgMIC13, Glu 221 and 223 are replaced by positively charged Arg and Lys residues, which would not only reduce any electrostatic repulsion but provide opportunities for the formation of specific charge-charge interaction with the second sialic acid.

We also derived homology models for the MAR domains of NcMIC1 based on the crystal structure of TgMIC1-MARR. The preference of NcMIC1-MARR for the two sialyl Le\(^\text{x}\)-related probes sulphated in the 6-position of galactose could be explained by the presence of a positive charged Lys (K176) in NcMIC1, which is replaced by an Ala in TgMIC1 (Fig. 7C). In our homology model K176 is spatially closely positioned to the 6-position of galactose and could form a specific charge-charge interaction with the negative charged sulphate. K176 in NcMIC1 is not conserved in TgMIC13 but is present in NcMCP2.

8. Gangliosides and 3’SiaLacNAc are potential determinants for *T. gondii* binding to host cells

Earlier data from carbohydrate microarray analyses on the binding preferences of TgMIC1-MARR suggested that gangliosides GD1a and GT1b are among the most potent binding molecules (Blumenschein et al, 2007); these were also bound by TgMIC13 in the carbohydrate microarray analyses (suppl. Table I). In order to determine whether gangliosides are able to compete with the authentic host cell receptor(s) for binding of
TgMIC1 and TgMIC13, we performed cell-binding assays using gangliosides GD1a and GT1b incorporated into liposomes as competitors. Binding of recombinant TgMIC1 to the cells was hardly detectable in the presence of 500µM GD1a or GT1b (Fig. 8A). Binding of TgMIC13 was also abolished in the presence of 500µM GD1a, and the presence of GT1a had a clear inhibitory effect (although weak binding could be detected even at 2mM GT1b upon long exposure of the blot) (Fig. 8A). Lipid-linked oligosaccharide ligands displayed on liposomes can be much more potent inhibitors than free oligosaccharides due to the multivalent presentation. Therefore the nominal concentrations of the liposome inhibition assays can not be compared with concentrations of free oligosaccharides used in our cell binding assays.

Binding assays using C6 rat glioma cells deficient in complex gangliosides (such as GD1a and GT1b) showed that both TgMIC1 and TgMIC13 bind to these cells (Fig. 8B) implying – in agreement with our carbohydrate microarray data - that glycoconjugates other than complex gangliosides can be bound by these molecules. As mentioned above, several of the probes in this array strongly bound by TgMIC1 and TgMIC13 are based on the 3’SiaLacNAc sequence. In order to determine if such high affinity is physiologically relevant, we examined if this interaction could be reproduced in a cell-binding assay. Interestingly, binding of TgMIC1 and TgMIC13 was abolished by concentrations of free 3’SiaLacNAc as low as 1mM and 5mM respectively, whereas increasing concentrations of LacNAc had no effect (Fig. 8C). In a parallel assay, the competition of NANA for TgMIC13 binding was reproducible, suggesting that the inhibitory concentrations of free 3’SiaLacNAc for TgMIC1 and TgMIC13 are lower than those of free NANA required for equivalent binding inhibition (Blumenschein et al, 2007). This increased affinity can be explained by an additional contact observed in the crystal structure of TgMIC1-MARR in complex with 3’SiaLacNAc between the galactose and the protein which is involved in the preference of the protein for 2-3 linked sialylated glycoconjugates (Garnett et al, 2009).
Discussion

Several studies and our previous results have highlighted the critical contribution of carbohydrate recognition on the host cell surface during invasion by *T. gondii*. TgMIC2, a member of the thrombospondin-related anonymous protein (TRAP) family, conserved throughout the Apicomplexa, is an essential component for parasite motility and invasion and has been shown to interact with heparin via its integrin-A-like domain upon multimerization (Harper et al, 2004). TgMIC3 and TgMIC8 are part of another major complex involved in invasion. Dimerization of a chitin-binding-like domain present in TgMIC3 and TgMIC8 allows host cell binding and has been associated with parasite virulence (Cerede et al, 2002; Cerede et al, 2005). The TgMIC1-4-6 complex, critical for invasion and virulence in mice, binds sialylated glycoconjugates, and a synergy-effect has been demonstrated between this complex and TgMIC3 (Blumenschein et al, 2007; Cerede et al, 2005).

In this study we have characterized key parasite molecular players involved in sialic acid-dependent host cell invasion by *T. gondii*. The salient findings in this investigation are first, the characterization of TgMIC1-4-6 complex as one important player in sialic acid-dependent host cell invasion, second, the reliance of the parasite on an additional sialic acid-binding lectin for invasion, identified as TgMIC13 and third, the determination of distinct sialic acid-dependent binding specificities for three members of a novel family of MAR domain containing proteins (MCPs) conserved in coccidians. In *T. gondii* all the four MCPs are expressed in tachyzoites. Among them, only TgMIC1 and TgMIC13 possess the sequence characteristics of a sialic acid-binding MAR domain, and only these specifically recognize sialyl oligosaccharides. The results of our study now add a hypothetical TgMIC13-containing complex to the list of carbohydrate binding parasite surface complexes.
Distinct binding specificities might determine tissue tropism of parasitic infections

With carbohydrate microarray technology, we examined details of carbohydrate-binding specificities of TgMIC13, in comparison with MAR regions of TgMIC1 and NcMIC1. The preponderant binding activity of all three proteins was to α2-3-linked sialyl probes. However, the relative binding intensities to specific sialyl probes were quite different for the three proteins. TgMIC13 bound to two sialyl oligosaccharide probes, namely the 4-O-acetylated sialyllactose and the α2-9-linked disialyl sequences, which were not recognized 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). There is so far limited information on the occurrence of 2-9-linked sialic acid in animals. It has been reported as a component on a murine neuroblastoma cell line (Inoue et al, 2003) and a human embryonal carcinoma-cell line (Fukuda et al, 1985). A special feature of NcMIC1 as revealed by carbohydrate microarray analyses is the strong binding to the two sulphated sialyl Le^x^-related probes. Sulphation was previously reported to play a role in cell surface binding by NcMIC1 (Keller et al, 2002); in that study, glycosaminoglycans were proposed to be targeted by NcMIC1; however the influence of sialic acid was not investigated. The distinct binding specificities of the three MIC proteins might have implications for tissue tropism, and the cellular targets for these proteins warrant further investigation.

Our results suggest that TgMIC1 and TgMIC13 recognize sialic acid on several types of glycoconjugates with differing affinities. In general 3'SiaLacNAc sequences are bound with high affinity by TgMIC1 and TgMIC13. This is in marked contrast to the *P. falciparum* erythrocyte binding antigen 175 (PfEBA175), which binds selectively to a unique sialylated glycoprotein, glycophorin A (Orlandi et al, 1992; Tolia et al, 2005). As our results suggest cooperativity between TgMIC1 and TgMIC4 in receptor binding by the TgMIC1-4-6 complex, we cannot exclude the possibility that TgMIC4 discriminates further in receptor
recognition and that some of the oligosaccharide sequences proposed are actually not recognized by this complex. Identification of the host cells receptor(s) recognized by TgMIC1 and TgMIC13 will be necessary to resolve this issue. Given the ubiquitous distribution of sialylated glycoconjugates in tissues, the binding preferences of TgMIC1 and TgMIC13 are likely to be important for parasite dissemination. This view is supported by the fact that TgMIC1 contributes to parasite virulence in mice (Cerede et al, 2005). The finding that gangliosides GD1a and GT1b are able to compete with host cell receptors for TgMIC1 and TgMIC13 binding is of particular interest for parasite biology regarding the colonization of the ganglioside-rich brain tissue prior to cyst formation. In addition, considering the fact that the MAR family is restricted to enteroparasites, these adhesins might be of importance in the context of the natural route of infection via the intestine. To our knowledge no study has so far addressed the importance of receptor-ligand interactions for invasion of the midgut epithelium.

The role of TgMIC13 in host cell invasion by T. gondii

To contribute to host cell invasion, TgMIC13 needs to be targeted accurately to the micronemes and then delivered in a timely fashion, firmly anchored on the parasite surface. Since TgMIC13 does not contain a predicted membrane spanning domain or GPI-anchor motif, it likely belongs to a yet undefined complex. Expression of TgMIC13 in the mic1ko and mic6ko strains ruled out any interaction with the TgMIC1-4-6 complex. A recent study on TgMIC3 has shown that a signal peptide and an EGF domain can be sufficient for microneme targeting of soluble proteins, presumably by the means of an unidentified escorter (El Hajj et al, 2008; Kessler et al, 2008). However, TgMIC13 does not possess EGF modules, nor does it contain a galectin-like domain like TgMIC1, which precludes a similar architecture for the putative TgMIC13-containing complex. Unravelling the mechanism of TgMIC13 sorting to the micronemes must await the identification of its escorter protein, as described for other
soluble microneme proteins (Meissner et al, 2002; Reiss et al, 2001). Despite several attempts, it has not been possible to disrupt the $TgMIC13$ gene, even in the delta-ku80 parasite line (Fox et al, 2009; Huynh & Carruthers, 2009), which strongly suggests that this gene is crucial for parasite propagation.

**Concluding remarks**

The presence of the MAR domain containing proteins in *Toxoplasma, Neospora, Sarcocystis* and *Eimeria* parasites and its absence in *Plasmodium, Theileria* and *Cryptosporidium* suggests that this domain is exclusively conserved among the Coccidia. We hypothesize that sialylated receptors may be exploited as a common target by a subset of MCPs carrying the appropriate binding motif in this group of enteroparasites. As suggested in this study, not all MCPs could bind to sialic acid or even to host cells which raise questions regarding their alternative functions. We have identified a complete set of homologues of *T. gondii* MCPs in *N. caninum*, a parasite causing significant economic loss by infecting cattle. Similar to *T. gondii*, *N. caninum* is also a promiscuous pathogen, infecting very different cell types. It is tempting to speculate that this novel family of lectins is instrumental to infection of such a broad range of cells.
Materials and Methods

Generation of *T. gondii* mutant parasite strains

All *T. gondii* strains were grown in human foreskin fibroblasts (HFF) or Vero cells in DMEM (GIBCO) supplemented with 10% fetal calf serum (FCS), 2 mM glutamine and 25 mg/mL gentamicin. The RH-strain is referred to as “wild-type”.

The *mic1ko* parasite strain, generated previously (Reiss et al, 2001), was stably complemented with linearized plasmids pM2MIC1myc, pM2MIC1\textsuperscript{T220A}myc, pM2MIC1\textsuperscript{T126A,T220A}myc and pROP1mycMIC1-GLD coding for the expression of TgMIC1myc, TgMIC1\textsuperscript{T220A}myc, TgMIC1\textsuperscript{TTAA}myc and mycTgMIC1-GLD (encompassing amino acids 299-456 of TgMIC1) respectively using a standard electroporation transfection protocol with restriction enzyme mediated insertion (REMI). For selection plasmids were cotransfected with p2854\_DHFR or pTUB5-CAT (carrying the pyrimethamine and the chloramphenicol resistance marker respectively) at a 5:1 ratio. pM2MIC1\textsuperscript{T220A}myc was generated from pM2MIC1myc using the Quickchange kit (Stratagene) and primers TgMIC1-20\_1731 and TgMIC1-21\_1732. pM2MIC1\textsuperscript{T126A,T220A}myc was obtained by replacing the fragment between restriction sites NdeI and EcoNI in pM2MIC1myc with the equivalent region in plasmid pPICZ\textalpha-TgMIC1\textsuperscript{NTT126A,T220A} (see (Blumenschein et al, 2007)). To obtain a clonal line, parasites were cloned at least twice by limiting dilution from the drug-resistant pool of parasites obtained from transfection and selection.

Cloning of *T. gondii* MCPs

Restriction enzymes were purchased from New England Biolabs. All primers are listed in suppl. Table 2. Accession numbers are listed in Table 1. TgMIC13, TgMCP3 and TgMCP4 were amplified by PCR from a *T. gondii* tachyzoite cDNA pool. TgMIC13 with or without
the signal peptide was amplified using primer pairs MIC1/2-5/1047 and MIC1/2-6/1048 or MIC1/2-1/1147 and MIC1/2-2/1148 respectively. TgMCP3 was cloned using primers MIC1/3-7_1981 and MIC1/3-7_1982. To obtain the coding sequence for TgMCP4 with or without the signal peptide, primer pairs TgMIC1/4-7_1983 and TgMIC1/4-7_1984 as well as TgMIC1/4-3_1847 and TgMIC1/4-4_1848 were used for amplification respectively. The fragments were cloned into pGEM and sequenced.

The sequence encoding full-length TgMIC13 was digested with EcoRI and SbfI and sub-cloned into pTUB8TgMLCTy_HX between EcoRI and NsiI restriction sites.

pTUB8TgMLCTy_HX was cut with EcoRI, blunt ended with endonuclease and cut again with NsiI prior to insertion of full-length TgMCP3 coding sequence cut from pGEM-TgMCP3 with EcoRV and PstI. TgMCP3 without signal peptide (SP) was cloned into pROP1mycMIC1-GLD in between NsiI and PacI (blunt end cloning) resulting in pROP1mycTgMCP3(3430).

Full-length TgMCP4 was cloned into pTUB8TgMLCTy_HX using EcoRI and NsiI restriction sites. The sequence corresponding to TgMCP4 without SP was sub-cloned into pROP1mycMIC1-GLD using NsiI and PacI restriction sites.

Constructs were used in transient transfection experiments in either RHΔHx, *mic1ko* or *mic6ko* parasites. Stable parasite lines were derived from transfection of the pTUB8TgMLCTy_HX based vectors carrying the mycophenolic acid/xanthine resistance marker into the RHΔHx strain.

**Expression and purification of MCPs from *E. coli***

TgMIC1 (aa 17-262), TgMIC13 (aa 23-468), TgMCP3 (aa 54-565), TgMCP4 (aa 526-1016) and NcMIC1 (aa 17-259) were expressed using the pET32Xa/LIC plasmid (Novagen) in *E. coli* Origami (DE3) (Stratagene), at 28° C (Saouros et al, 2007). Protein expression was induced with 800 μM isopropyl β-D-thiogalactopyranoside. The hexahistidine-thioredoxin
fusion proteins were purified using nickel-nitrilotriacetic acid HISBind resin (Novagen). For glycoarray analysis proteins were concentrated into PBS, pH 7.2.

**Expression and purification of MCPs from *P. pastoris***

*P. pastoris* transformation and expression was performed using pPICZα-based plasmids according to the protocols supplied with the *Pichia* expression kit (Invitrogen). Transformation of the supplied host strain GS115 was performed by electroporation following linearization of the vector with PmeI, SacI or DraI. To inhibit glycosylation expression was occasionally carried out in the presence of 10µg/ml tunicamycin. Recombinant TgMIC13 (aa 23-468) was either used directly in the form of culture supernatant or purified on NiNTA (Qiagen) and concentrated into 20mM NaH₂PO₄/Na₂HPO₄, pH 7.3. TgMCP3 (aa 54-565) and TgMCP4 (aa 526-1016) were not secreted, but soluble α-factor fusion-protein was obtained by lysis of cells in 50mM NaH₂PO₄/Na₂HPO₄, pH 7.4, 5% Glycerol, 1mM PMSF.

**Preparation of Excreted Secreted Antigen fraction (ESA)**

*T. gondii* tachyzoites freshly lysed from their host cells were harvested by centrifugation at 240g for 10 minutes and washed twice in IM (DMEM / 3% FBS / 10mM HEPES) prewarmed to 37°C. A pellet of 2,0 - 4,88 x 10⁸ parasites was resuspended in 1 ml IM, an aliquot of 50 µl was taken as reference standard to allow estimation of the degree of secretion, and microneme secretion was stimulated by adding 10 µl of 100% EtOH to the remaining 950 µl. The sample was incubated for 10 minutes at RT, then 40 minutes at 37°C and finally cooled down to 0°C in an ice-water bath for 5min. Parasites were pelleted at 1000g, 4°C for 5 min. The supernatant was transferred to a new tube and centrifuged again for 5min, 4°C at 2000g. The supernatant (ESA) was collected and stored at -80°C.
**Cell binding assays**

These were performed as described previously (Blumenschein et al, 2007). GD1a di-sodium salt and GT1b tri-sodium salt were purchased from Alexis Biochemicals, L-α-phosphatidylcholine, cholesterol, NANA and glucuronic acid from Sigma, 3’Sia-LacNac and LacNac from Dextra, and α-2,3,6,8- Vibrio cholerae neuraminidase from Roche. Stock solutions of NANA and glucuronic acid in PBS were adjusted to pH 7.0.

Liposome inhibition assays were performed as follows. In glass vials, the lipid-linked oligosaccharide (here gangliosides GD1a and GT1b) were dispensed, together with carrier lipids, L-α-Phosphatidylcholine and cholesterol (all solutions in methanol), in a molar ratio of 1:5:5. The methanol was evaporated using a gentle stream of nitrogen and the samples dried completely by lyophilisation for 30 minutes. PBS was added to vials to give a nominal ganglioside concentration of 4 mM. Vials were sonicated in a water bath at 37°C for 30 min. Formation of liposomes was checked by dynamic light scattering.

**Comparison of invasion efficiency of different strains**

Comparison of different *T. gondii* strains for invasion efficiency was done using an RH-2YFP strain (Gubbels et al, 2003) as internal standard for parasite fitness. The details were described previously (Sawmynaden et al, 2008). Total number of parasite vacuoles and RH-2YFP parasite vacuoles were counted on 20 microscopic fields on each IFA-slide with a minimum of 440 vacuoles in total per slide. Only vacuoles containing at least two parasites were counted to be sure not to count extracellular parasites that resisted the washing. Each experiment was repeated at least three times. Statistical analysis was performed using PRISM.

**Microarray analyses of recombinant MIC proteins**

The sialyl oligosaccharide microarrays were generated with 88 lipid-linked oligosaccharide probes (Supplementary Table 1), which were arrayed in duplicate on nitrocellulose-coated
glass slides at 2 and 5 fmol per spot using a non-contact instrument (Palma et al, 2006). Analysis of carbohydrate-binding of the recombinant His-tagged MIC proteins was performed essentially as described (Blumenschein et al, 2007). In brief, each His-tagged MIC protein was precomplexed with mouse monoclonal anti-poly-histidine and biotinylated goat anti-mouse IgG antibodies (Sigma) in a ratio of 1:2.5:2.5 (by weight), and overlaid onto the arrays at 40 µg/ml for TgMIC1-MARR and TgMIC13, and 20 µg/ml for NcMIC1-MARR. Binding was detected using Alexa 647 fluor conjugated streptavidin (Molecular Probes). Microarray data analysis and presentation were carried out using dedicated software (M. S. Stoll, unpublished). The binding to oligosaccharide probes was dose related, and results of 5 fmol per spot are shown.

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**Author contributions**

NF, DS and MJB designed the study. NF did sequence and phylogenetic analyses and performed invasion assays. NF and JDS generated parasite lines, produced recombinant proteins and performed cell binding assays. JDS conducted TgMIC13 knockout attempts, produced parasite ESA and generated TgMIC13 Abs. MK provided the synthetic glycolipids for the microarrays. YL and ASP designed and performed microarray experiments. YL analysed the microarray data. EL and SS produced recombinant NcMIC1. SM performed the modelling studies. NF and DS wrote the paper with contributions from YL, TF, SM and MJB.

**Figure legends**

**Fig. 1**

A cooperative function for the sialic acid binding TgMIC1-MAR domains and TgMIC4 in host cell invasion by *T. gondii.*

A. Schematic summarizing the domain organisation of the components of the TgMIC1-4-6 complex in the wt and *mic1ko/MIC1myc* complemented strain. B. Western blot analysis of parasite lines expressing TgMIC1 mutant proteins on the *mic1ko* background. C, D and E. TgMIC1 mutant proteins expressed on the *mic1ko* background were assessed for their ability to substitute for TgMIC1 and target the components of the TgMIC1-4-6 complex to the micronemes. IFA was performed on intracellular parasites multiplying in their vacuole. TgAMA-1 is used as a micronemal marker independent of the TgMIC1-4-6 complex. Scale bars, 1 µm. A schematic summarizes the association/dissociation of the components of the
TgMIC1-4-6 complex in the different strains. An asterisk indicates a Thr to Ala substitution in the sialic acid binding site of the MAR domain. **F.** Comparison of host cell invasion efficiency by the various *T. gondii* mutant strains using an RH-2YFP strain as internal standard for parasite fitness. Error bars, standard deviation.

**Fig. 2**

Cell invasion assays using the *T. gondii* mic1ko strain demonstrate the existence of at least one more sialic acid parasite lectin. **A.** Invasion by *mic1ko* parasites in absence and presence of 10 mM and 20 mM free NANA or galactose. **B.** Assay comparing invasion of *mic1ko* parasites into host cells (HFFs) pre-treated or not with neuraminidase. Error bars, standard deviation.

Note that, compared to the parental strain the *mic1ko* shows a 50% reduced invasion phenotype (**Fig. 1F**) but its invasion efficiency was set to 100% here.

**Fig 3**

A family of MAR domain containing proteins in Apicomplexans

**A.** Schematic of the domain organisation of various MCPs. MAR domain type I (turquoise), MAR domain type II (dark green), MAR domain type II extension “the beta-finger” (light green), galectin-like domain (orange), repeat region (blue strips). The presence of a Thr in a MAR domain in an equivalent position to those critical for sialic acid binding in TgMIC1-MARR is indicated by a black T. The fourth MAR domain in TgMCP4 contains this Thr, however the sequence context does not fit with it being indicative of a potential sialic acid binding site; therefore the T is in parentheses.

**B.** Phylogenetic relationship of MCPs from *T. gondii*, *N. caninum* and *E. tenella*. All bootstrap values are >80. Since the domain structure of the different proteins varies, only sequence corresponding to the first two predicted MAR domains of each protein was used for
the analysis. Sequences were aligned in ClustalX and alignment positions containing gaps in >50% of the sequences were excluded from phylogenetic analyses. Phylogenetic analyses were carried out using POWER (http://ca.expasy.org/, neighbor-joining distance method, bootstrapping with 1000 replicates). Phylogenetic trees were generated using TREEVIEW.

**Fig. 4**

Characterisation of TgMIC13 (TgMCP2) in *T. gondii*.

**A.** Sub-cellular localisation by IFA in wild-type (top, confocal images) and in wt/TgMIC13ty parasites (bottom). **B.** Western blot analysis of endogenous and epitope-tagged TgMIC13 in wild-type and wt/TgMIC13ty parasites. An extract of HFF cells was also loaded as a control. **C.** Assessment of TgMIC13 solubility by fractionation (top). Cell binding assays using ESA from wt/TgMIC13ty parasites (bottom). S: supernatant, W: last of 4 washes, CB: cell bound fraction. **D.** Transient transfection of TgMIC13ty into the *mic1ko* and the *mic6ko* show correct localisation of TgMIC13ty to the micronemes. Scale bars, 1µm.

**Fig. 5**

Binding characteristics of recombinant TgMIC13.

**A.** Cell binding assays using recombinant TgMIC13myc, TgMIC13myc and alpha-factor-TgMCP3myc fusion protein produced in *P. pastoris*; +tu/-tu: protein expressed in the presence/absence of tunicamycin. Top panel: Western blot indicating the relative concentration of the proteins in supernatants (S) used for the assay. Middle panel: Cell bound fractions (CB) probed for bound protein. Bottom panel: Control for use of equivalent amounts of cell material in each experiment. **B.** Pre-treatment of cells with neuraminidase abolishes binding by TgMIC1 and TgMIC13. S: supernatant, W: last of 4 washes, CB: cell bound fraction, C: background control. **C.** Binding of TgMIC13 is strongly reduced in cell binding assays by competition with free NANA but not glucuronic acid. **D.** Binding of TgMIC1 and
TgMIC13 to various CHO cell lines (K1: wt strain, 2: lec2 mutant with strong reduction in sialic acid surface expression, A and B: pgsA745 and pgsB618 mutants deficient in glycosaminoglycan synthesis, D: pgsD677 mutant deficient in two glycosyltransferase activities). Only the cell bound fractions of the assay are shown.

Fig. 6
Carbohydrate microarray analyses of recombinant TgMIC1-MARR expressed in *E. coli* (panel A), TgMIC13 expressed in *P. pastoris* (panel B) and NcMIC1-MARR expressed in *E. coli* (panel C) using 88 lipid-linked oligosaccharide probes printed on nitrocellulose-coated glass slides. Numerical scores of the binding signals are means of duplicate spots at 5 fmol/spot (with error bars). The complete list of probes, their sequences and binding scores are in Supplementary Table I. The binding signal for probe 88 was saturated and could not be accurately quantified (asterisk in B).

Fig. 7
**A, B.** Two orientations for the superposition of the homology model for the second MAR domain from TgMIC13 created using swiss-model (Bordoli et al, 2009) and the crystal structure of TgMIC1-MARR. TgMIC1 shown in cyan and TgMIC13 in violet; 3’ sialyl-**N**-acetyllactosamine is shown in an orange stick representation. Key side chains are drawn as stick representations minus attached protons and labelled with sequence position. **C.** Superposition of the homology model for the second MAR domain from NcMIC1 created using swiss-model (Bordoli et al, 2009) and the crystal structure of TgMIC1-MARR. TgMIC1 shown in cyan and NcMIC1 in violet; 3’ sialyl-**N**-acetyllactosamine is shown in an orange stick representation. Key side chains are drawn as stick representations minus attached protons and labelled with sequence position.

Fig. 8
Cell binding assays using recombinant TgMIC1myc and TgMIC13myc expressed in \textit{P. pastoris}. I: input, W: last of 4 washes, CB: cell bound fraction, C: background control. Only the cell bound fractions are shown in panels A and C. A. Gangliosides GD1a and GT1b incorporated into liposomes compete with the host cell receptor(s) for binding to TgMIC1myc and TgMIC13myc. B. Cell binding assay on HFFs and C6 rat glioma cells using TgMIC1myc and TgMIC13myc. C. Free 3’SiaLacNac, LacNac and NANA used as competitors in cell binding assays with TgMIC1myc and TgMIC13myc.

**Table I**

Proposed orthologous relationships and Genebank (and toxoDB/geneDB) accession numbers for various MCPs.

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<tr>
<th>(T. \text{gondii})</th>
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<th>(S. \text{neurona})</th>
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Supplementary Materials and Methods

Immunofluorescence Analysis

IFA was carried out on intracellular parasites grown in HFFs seeded on glass coverslips. After inoculation of parasites, the cells were incubated for 24-36 hours at 37°C before fixation to let the parasites divide within their vacuoles and form rosettes. Fixation is routinely done with 4% paraformaldehyde for 20 min, followed by 3 min incubation with 0.1 M glycine in PBS to quench the reaction. For detection of dense granule proteins mild fixation conditions (10 min incubation in 4% paraformaldehyde) are used. Fixation and all further manipulations are carried out at room temperature. Fixed cells were permeabilised with 0.2% Triton-X100 in PBS for 20 min and blocked in 2% bovine serum albumin, 0.2% Triton-X100 in PBS for 20 min. The cells were then stained with primary antibodies followed by secondary antibodies Alexa488- or Alexa594-conjugated goat anti-mouse or goat anti-rabbit IgGs (Molecular Probes). After 4,6-diamidino-2-phenylindole (DAPI) staining, slides were mounted in Fluoromount G (Southern Biotech) and stored at 4°C in the dark. Micrographs were obtained on a Zeiss Axioskop 2 equipped with an Axiocam color charge-coupled device camera. Images were recorded and treated on computer through the AXIOVISION™ software. ADOBE PHOTOSHOP (Adobe Systems) was used for processing of images. Confocal images were collected with a Leica laser scanning confocal microscope (TCS-NT DM/IRB) using a x63 Plan-Apo objective with numerical aperture (NA) 1.40. Optical sections were recorded at 250 nm per vertical step and four times averaging.

Cell invasion assays in the presence of carbohydrate inhibitors

These were done as described previously (Blumenschein et al, 2007). Two independent experiments were performed in triplicate. Numbers of extracellular (attached) and
intracellular (invaded) parasites were counted in three microscopic fields respectively. For the untreated controls 75 parasites were counted per microscopic field on average. Invasion efficiency of the untreated parasites was set to 100%.

**Cell invasion assays with neuraminidase treatment**

These were done as described previously (Blumenschein et al, 2007) using *Vibrio cholerae* neuraminidase (Calbiochem). Three experiments were carried out in parallel and evaluated in blind manner. According to the staining, numbers of extracellular (attached) and intracellular (invaded) parasites were counted in ten microscopic fields respectively. A total of 80 parasites per microscopic field were counted on average and invasion of the untreated sample was set to 100%. Enzyme activity was monitored in parallel in a protein cell binding assay using recombinant TgMIC1myc.

**Fractionation experiments**

2.0 - 4.88 x 10^8 *T. gondii* tachyzoites freshly egressed from their host cells were harvested by centrifugation at 240g, RT for 10 minutes and the pellet was resuspended in 1ml 1×PBS by rotating 1h at 4°C. After repeated freezing/thawing and sonication, the solution was centrifuged at maximum speed, for 30min, 4°C, and the supernatant was collected (fraction soluble in 1×PBS). The pellet was then resuspended in 1ml 1×PBS/0.3% Triton by rotating 1h, 4°C. After centrifugation, the supernatant was collected (fraction soluble in 1×PBS/0.3% Triton) and the pellet was resuspended in 1ml 1×PBS/0.2M Na₂CO₃, by rotating 1h, 4°C. After centrifugation, the supernatant was collected (fraction soluble in 1×PBS/0.2M Na₂CO₃). The supernatant and pellet fractions were run in SDS-PAGE and analyzed by western-blotting.
Cloning into expression vectors

Sequences corresponding to TgMIC13 (aa 23-468), TgMCP3 (aa 54-565) and TgMCP4 (aa 526-1016) were cloned into pET32-Xa/LIC (Novagen) *E. coli* and pPICZα *Pichia* expression vectors. The coding region for NcMIC1 (aa 17-259) was amplified from pCR-XL-TOPO-NcMIC1 using primers NcMIC1-f and NcMIC1-r and inserted into pET32-Xa/LIC.

The TgMIC13 coding sequence was inserted into pET32-Xa/LIC according to the manufacturer’s instructions. For expression from pPICZα in *P. pastoris*, TgMIC13 was cloned in between EcoRI and XbaI restriction sites.

The TgMCP3 (aa 54-565) coding sequence was reamplified from pGEM-TgMCP3 using primer pair TgMIC1.3_2285 and TgMIC1.3_2286 and cloned into pPICZα in between AvrII and NotI and into pET32-Xa/LIC-ROM6 in between NsiI and BamHI restriction sites.

TgMCP4 lacking the signal peptide was sub-cloned from pGEM-TgMCP4 into pPICZα between EcoRI and NotI sites. Similar, the fragment coding for TgMCP4 (aa 526-1016) amplified from pGEM-MIC1.4 with primers TgMIC1/4-9_2287 and TgMIC1/4-4_1848 was digested with EcoRI and NotI and inserted into the pPICZα *Pichia* expression vector. For expression in *E. coli*, the same primers were used and the fragment was cloned into a pET-Xa/LIC-derived vector (pET32-Xa/LIC-ROM6) which allows for cloning of sequences into the expression locus between EcoRI and PacI restriction sites.

Generation of Antibodies

Antibodies against the TgAMA1 C-terminus were raised in rabbits (EUROGENTEC, Belgium). The protein was expressed in *E. coli* BL21 strain as GST fusion from a pGEX-4T1 expression vector (pGEX-TgAMA-1tail) and purified on Glutathione-Sepharose (Amersham) according to the manufacturer’s instructions. Polyclonal antibodies against TgMIC13 were
raised in rabbits (28 days immunization protocol, EUROGENTEC, Belgium) using full-length recombinant TgMIC13 expressed as described above in *P. pastoris*.

**Supplementary figure S1**

Stable expression of MIC1myc in the *mic1ko* background. The ability of the epitope-tagged protein to substitute for MIC1 and bring the components of the TgMIC1-4-6 complex to the micronemes is assessed by IFA on intracellular parasites. AMA-1 is used as a micronemal marker which is independent of the TgMIC1-4-6 complex. Most of TgMIC1myc is targeted to the micronemes; however, as previously reported overexpression causes some TgMIC1myc protein to be mistargeted. Scale bars, 1 µm.

**Supplementary figure S2**

Alignment of MAR domain containing proteins (MCPs) from *T. gondii*, *N. caninum*, *S. neurona* and *E. tenella*. The position of the sialic acid binding motif relative to TgMIC1-MARR is boxed in red. Amino acid residues in TgMIC1_M2 critical for sugar binding and binding preference are marked with an asterisk (F169, A176, R178, K216, H218, T220, E206, E207). The aligned sequences correspond to the following amino acid stretches of the full-length sequences: TgMIC1_M1 (aa 35-143), M2 (aa 144-254); NeMIC1-M1 (aa 33-141), M2 (aa 142-252); TgMIC13_M1 (aa 51-154), M2 (aa 155-263), M3 (aa 274-375), M4 (aa 376-468); NeMCP2_M1 (aa 51-154), M2 (aa 155-263), M3 (aa 274-375), M4 (aa 376-468); TgMCP3_M1 (aa 118-222), M2 (aa 223-328), M3 (aa 339-439), M4 (aa 440-542); NeMCP3_M1 (aa 125-228), M2 (aa 229-334), M3 (aa 345-445), M4 (aa 446-550); TgMCP4_M1 (aa 554-658), M2 (aa 659-763), M3 (aa 774-873), M4 (aa 874-981); NeMCP4_M1 (aa 390-494), M2 (aa 495-600), M3 (aa 611-711), M4 (aa 712-819); NeMCP5_M1 (aa 54-157), M2 (aa 158-270), M3 (aa 348-451); NeMCP6_M1 (aa 51-154),
M2 (aa 155-266); NcMCP7_M1 (aa 53-156), M2 (aa 157-266); SnMCP5_M2, M3 and SnMCP6_M1 (derived from SnEST4a79g11.y1, SnEST4a34d02.y1 and SnESTbab01e09.y1, SnEST4a69h07.y1); EtMIC3_M1 (aa 45-148), M2 (aa 153-274), M3 (aa 294-425), M4: aa 445-576, M5: aa 596-727, M6 (aa 747-886), M7 (aa 887-980); EtMCP2_M1 (aa 37-138).

**Supplementary figure S3**

Alignment of the full-length amino acid sequences from TgMIC13 with NcMCP2 (top) and from TgMCP3 with NcMCP3 (bottom). The *N. caninum* genes were annotated *in silico* based on EST-data and the considerable conservation between the apicomplexan proteins.

**Supplementary figure S4**

Alignment of the full-length amino acid sequences from TgMCP4 with NcMCP4 (top) and from NcMCP5 with the partial sequence of SnMCP5 derived from ESTs SnEST4a79g11.y1 and SnEST4a34d02.y1 (bottom). The *N. caninum* genes were annotated *in silico* based on EST-data and the considerable conservation between the apicomplexan proteins.

**Supplementary figure S5**

Full-length amino acid sequences from NcMCP6, NcMCP7 and EtMCP2 as annotated *in silico* by comparison with the corresponding EST data set.
Supplementary Table I
Oligosaccharide probes* investigated in carbohydrate microarray analyses, sorted by sialyl linkage and backbone sequence.

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**Neutral oligosaccharide probes**

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**2,3-linked sialyl oligosaccharide probes**

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Lacto-M-neotetraose and Lacto-N-tetraose-based

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25 GSC-31  NeuAcα3Galβ4GlcNAcβ3Galβ4Glc8-Cer36  2367  -  483
26 GSC-516B  NeuAcα3Galβ4GlcNAcβ3Galβ4Glc8-Cer36  42  -  -
27 C4U  NeuAcα3Galβ4GlcNAcβ3Galβ4Glc8-Cer36  20762  6698  10514
28 SA(3')-LNFP-II  NeuAcα3Galβ4GlcNAcβ3Galβ4Glc8-Cer36  1331  -  1406
29 SA(3')-LNFP-III  NeuAcα3Galβ4GlcNAcβ3Galβ4Glc8-Cer36  1060  -  440
30 GSC-105  NeuAcα3Galβ4GlcNAcβ3Galβ4Glc8-Cer36  91  -  61
31 GSC-64  NeuAcα3Galβ4GlcNAcβ3Galβ4Glc8-Cer36  346  -  249
32 GSC-472  NeuAcα3Galβ4GlcNAcβ3Galβ4Glc8-Cer36  -  -  -
33 GSC-268  NeuAcα3Galβ4GlcNAcβ3Galβ4Glc8-Cer36  4814  4248  27309
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35 GSC-269  NeuAcα3Galβ4GlcNAcβ3Galβ4Glc8-Cer36  5754  3528  5398
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Branched milk

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### 2,6-linked sialyl oligosaccharide probes

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### Lacto-N-neotetraose and Lacto-N-tetraose-based

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<th>NeuAcβ-3Galβ-3Galβ-4Glcβ-DH</th>
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136
61 SA(6')-LNFP-VI  NeuAcα-6Galβ-4GlcNAcβ-3Galβ-4Glc-DH  158  -  106

Branched milk

62 MSLNH  NeuAcα-6Galβ-4GlcNAcβ-6
          Galβ-3GlcNAcβ-3
          Galβ-4GlcNAcβ-6
                Galβ-4Glc-DH
              NeuAcα-6Galβ-4GlcNAcβ-3  94  -  -
                Galβ-4Glc-DH

63 MSLNnH-I  NeuAcα-6Galβ-4GlcNAcβ-6
          Galβ-3GlcNAcβ-3
          Galβ-4Glc-DH
                NeuAcα-6Galβ-3GlcNAcβ-3  268  -  43

64 DSLNnH  NeuAcα-6Galβ-4GlcNAcβ-6
          Galβ-3GlcNAcβ-3
          Galβ-4Glc-DH
                NeuAcα-6Galβ-3GlcNAcβ-3  333  -  161

65 MFMSLNnH  NeuAcα-6Galβ-4GlcNAcβ-3
          Fucα-3
          Galβ-4Glc-DH
                NeuAcα-6Galβ-3GlcNAcβ-3  41  -  -

N-glycan

66 A2(2-6)  NeuAcα-6Galβ-4GlcNAcβ-2Manα-6
            Manβ-4GlcNAcβ-4GlcNAc-DH  712  -  183
            NeuAcα-6Galβ-4GlcNAcβ-2Manα-3

2,3,2,6-linked sialyl oligosaccharide probes

O-glycan

67 DST  NeuAcα-3Galβ-3GalNAc-DH
        NeuAcα-6
                NeuAcα-6  2386  2525  4200

Lacto-N-neotetraose and Lacto-N-tetraose-based

68 DSLNT  NeuAcα-3Galβ-3GalNAcβ-3Galβ-4Glc-DH
          NeuAcα-6
                NeuAcα-6  5230  1296  1617

N-glycan

69 A3  NeuAcα-3Galβ-4GlcNAcβ-2Manα-6
          Manβ-4GlcNAcβ-4GlcNAc-DH
                NeuAcα-6Galβ-4GlcNAcβ-2Manα-3  2345  26  855
                NeuAcα-6Galβ-4GlcNAcβ-2

2,8-linked sialyl oligosaccharide probes

Ganglioside-based

70 GD3  NeuAcα-8NeuAcα-3Galβ-4Glcβ-Cer  -  -  -
71 GD3-tetra  NeuAcα-8NeuAcα-3Galβ-4Glc-DH  -  -  112
72 GD3-tetra-AO  NeuAcα-8NeuAcα-3Galβ-4Glc-AO  99  -  15
73 GD2  NeuAcα-8NeuAcα-3Galβ-4Glcβ-Cer
          NeuAcα-8NeuAcα-3
                NeuAcα-8NeuAcα-3  13  -  -
74 GD1b  NeuAcα-8NeuAcα-3Galβ-3GalNAcβ-4Galβ-4Glcβ-Cer
          NeuAcα-8NeuAcα-3
                NeuAcα-8NeuAcα-3  6  -  -
75 GQ1b  NeuAcα-8NeuAcα-3
          NeuAcα-8NeuAcα-3
**Polysialyl**

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**2,3:2,8-linked sialyl oligosaccharide probes**

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**2,9-linked sialyl oligosaccharide probe**

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*a The oligosaccharide probes are all lipid-linked, and are from the collection assembled in the course of research in Glycosciences Laboratory. DH, designates NGLs prepared from reducing oligosaccharides by reductive amination with the amino lipid, 1,2-dihexadecyl-sn-glycero-3-phosphoethanolamine (DHPE; AO, NGLs prepared from reducing oligosaccharides by oxime ligation with an aminooxy (AO) functionalized DHPE (Liu et al., Chem. Biol. 14, 847–859, 2007); Cer, natural glycolipids with various ceramide moieties; Cer36, synthetic glycolipids with ceramide having a total of 36 carbon atoms. Neu, denotes de-N-acetylated neuraminic acid.

*b* Pos, position in screening microarray.

*c* Signal less than 1.

*d* Major component.
**Supplementary Table II**

**Primer sequences**

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<th>Primer name</th>
<th>Primer sequence</th>
<th>Gene to be amplified</th>
<th>Target vector</th>
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<td>MIC1/2-5/1047 (sense)</td>
<td>5’-CGAATTCCCTTTTTTCGACAAAAATGTTGGGTGGTTTCCAGCTG-3’</td>
<td>TgMIC1</td>
<td>pTUB8TgMLCTy_HX</td>
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<tr>
<td>MIC1/2-6/1048 (antisense)</td>
<td>5’-CCCTGCAGGGCACTCTGTGAGGCGCTTTTCA TAGC-3’</td>
<td>TgMIC1</td>
<td>pTUB8TgMLCTy_HX</td>
</tr>
<tr>
<td>MIC1/2-1/1147 (sense)</td>
<td>5’-CCCCGAATTCATCCAGCTCGGCCGAGCATCG-3’</td>
<td>TgMIC1</td>
<td>pPICZα</td>
</tr>
<tr>
<td>MIC1/2-2/1148 (antisense)</td>
<td>5’-GGGTCTAGAATGCACTCTGTGAGGCGCTTTTCA TTC-3’</td>
<td>TgMIC1</td>
<td>pPICZα</td>
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<tr>
<td>TgMIC1.3_2285 (sense)</td>
<td>5’-cgatgcctcatctaggGATCCTTTTTGCAACAAAGGACC GAG-3’</td>
<td>TgMAR-CP3</td>
<td>pET32-Xa/LIC and pPICZα</td>
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<tr>
<td>TgMIC1.3_2286 (reverse)</td>
<td>5’-ccggatccgccggtgcatTAATCCCGGTAGCCTTTCGATCtc-3’</td>
<td>TgMAR-CP3</td>
<td>pET32-Xa/LIC and pPICZα</td>
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<tr>
<td>MIC1/3-7_1981 (sense)</td>
<td>5’-gatatctctctctaccaatATGGCGAAGCC-3’</td>
<td>TgMAR-CP3</td>
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<tr>
<td>MIC1/3-7_1982 (reverse)</td>
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<td>TgMAR-CP4</td>
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<td>TgMIC1/4-9_2287 (sense)</td>
<td>5’-cgatgcctgaattccAGTGCAACAAAGGACCCTGTGC-3’</td>
<td>TgMAR-CP4 (partial)</td>
<td>pPICZα and pET32Xa/LIC</td>
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<td>pPICZα and pROP1mycMIC1-GLD</td>
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<td>TgMAR-CP4</td>
<td>pPICZα, pET32Xa/LIC and pROP1mycMIC1-GLD</td>
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<td>TgMIC1-21_1732</td>
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<td>NeMIC1-f</td>
<td>5’-GGTAAGGGGTGGTGTCGCTGGGGCAAAAAACATACG GAGAAGGCGTCG 3’</td>
<td>NeMIC1</td>
<td>pET32Xa/LIC</td>
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References


Figure 1

A

WT and mic1ko/MIC1myc

B

MIC1myc

MIC1T220Amyc

MIC1TTAAmyc

mic1myc

mic1ko/MIC1GLD

C

myc

AMA1

merge

myc

MIC4

merge

myc

MIC6

merge

D

mic1ko/
MIC1T220Amyc

myc

AMA1

merge

myc

MIC4

merge

myc

MIC6

merge

E

mic1ko/
MIC1TTAAmyc

myc

AMA1

merge

myc

MIC4

merge

myc

MIC6

merge

F

invasion [%] vs. RH-YFP

wild-type

mic1ko

mic1ko/MIC1-GL3

mic1ko/MIC1T220Amyc

mic1ko/MIC1TTAAmyc

mic1ko/MIC1GLD
Figure 2

A

B

invasion %

control 10 mM NANA 20 mM NANA 10 mM galactose 20 mM galactose

invasion %

- + Neuraminidase
Figure 3

A

B

146
Figure 4

A

B

C

D

wt

merge

wt/MIC13ty

wt

HFF

wt/MIC13ty

anti-MIC13

anti-MIC4

anti-ty

D

mic1ko

mic6ko
Figure 5

A

Supernatants: anti-myc

CB-fractions: anti-myc

CB-fractions: anti-tubulin

B

Mic1myc Mic13myc

W C5 C6 W C6 C

-+ neuraminidase

anti-myc

anti-tubulin

C

Mic13myc + x mM NANA

0 1 3 10 30 50 c

anti-myc

anti-tubulin

D

Mic13myc on CHO-cell lines

K1 2 A B D c

anti-myc

anti-tubulin
Figure 7

A

B

C
Figure 8

A

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<tr>
<th>MIC1myc</th>
<th>+ x μM GD1a</th>
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- anti-myc
- anti-tubulin

B

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<th>MIC1myc</th>
<th>HFF</th>
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<td>HFF</td>
<td>S W CB C</td>
<td>W CB C</td>
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<tr>
<td>C6</td>
<td>S W CB C</td>
<td>W CB</td>
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- anti-myc
- anti-tubulin

C

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<th>MIC1myc</th>
<th>+ x mM 3' Sia-LacNac</th>
<th>+ x mM LacNac</th>
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- anti-myc
- anti-tubulin
Supplementary figure 1
Supplementary figure 2

type I MAR-domain

type II MAR-domain

---
Supplementary figure 3

TgMIC13/NcMCP2: 73% id., 86% sim.

TgMCP3/NcMCP3: 67% id., 75% sim.
Supplementary figure 5

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4. Discussion

A wide spectrum of pathogens including bacteria and protozoa has adapted to an intracellular lifestyle which presents several advantages, including accessibility to nutrients from the host cell and partial protection from the host immune system. These pathogens have developed numerous strategies to enter their respective host cells. Sialic acid is abundantly found on cell surface glycoproteins and glycolipids and is widely exploited by viruses and bacteria to enter host cells. The most prominent example is probably the binding of influenza viruses to airway epithelium (Suzuki, 2005). Carbohydrate-bearing receptors are also important for host cell invasion by protozoan parasites of the phylum Apicomplexa and in particular sialic acid is now recognized to critically contribute to host cell recognition by a variety of species. Glycophorins A, B, and C are sialoglycoproteins of human erythrocytes that serve as important and highly specific receptors for the *P. falciparum* DBL-EBP family ligands. Sialoglycophorins on RBCs seem also to play an important role in *Babesia* infections (Takabatake et al, 2007a; Takabatake et al, 2007b). In this study we have shown that sialic acid constitutes a major determinant for host cell invasion by *T. gondii* and have characterized TgMIC1 and TgMIC13 as the key molecular players involved in Sia-dependent host cell recognition.

4.1 Function of MAR containing proteins (MCPs) in *T. gondii*

The TgMIC1-4-6 complex has been formally demonstrated to function in host cell invasion (Cerede et al, 2005; Reiss et al, 2001) and all available data supports a role for a hypothetical TgMIC13 containing complex in invasion as well. Despite several studies on the TgMIC1-4-6 complex and our efforts on the characterization of TgMIC13 it appears still difficult to pinpoint the exact role of these adhesins. In particular the timing and coordination of the individual parasite ligand-host cell receptor interactions during the invasion process is so far poorly understood.
4.1.1 Connection of adhesive complexes to the actin-myosin motor

Previous studies on the characterization of the mic1ko failed to detect any significant defect in parasite attachment to host cells (Cerede et al, 2005) which is in agreement with our own observations. This might be due to the experimental conditions used; alternatively the adhesive function of the TgMIC1-4-6 complex could be critical during the penetration process. So far it was impossible to localize this complex or TgMIC13 during invasion. Motility and invasion in apicomplexan parasites is driven by the glideosome (Soldati-Favre, 2008). Similar to TgMIC2 (Jewett & Sibley, 2003), in vitro experiments show that TgMIC6 is able to connect to this motor machinery via interaction with the F-actin binding protein aldolase ((Zheng et al, 2009) and Sheiner et al., in revision). Therefore the TgMIC1-4-6 complex potentially provides a link between the host cell surface and the parasite motor. Whether this link is needed for reorientation, penetration or other mechanistic aspects of invasion is not yet known. However, our own unpublished results failed to detect an association of TgMIC6 with the motor machinery in the parasite during invasion. The interaction of TgMIC2 with aldolase relies on a tryptophan residue in the extreme C-terminus of the cytoplasmic tail (Buscaglia et al, 2003; Jewett & Sibley, 2003; Starnes et al, 2006) which is conserved in TgMIC12 and TgMIC6. A mic6ko strain was complemented with a mutant carrying a tryptophan to alanine substitution in this position. Subsequent invasion assays did not show any defect of the strain complemented with the mutant compared to a control strain complemented with a wild-type copy (data not shown). This result suggests that interaction of MIC6 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 TM-MICs capable of interacting with the glideosome making the interaction of TgMIC6 with aldolase redundant.

4.1.2 Function of TgMCP3 and TgMCP4

As we could not detect any carbohydrate binding for TgMCP3 and TgMCP4, their function remains so far a mystery. Parasite lines expressing epitope-tagged TgMCP3 and TgMCP4 were generated (Joana Santos, unpublished results). Both C-terminally tagged proteins were detected as a single species on Western Blot. In contrast to the majority of microneme proteins that target to the organelle when expressed as second copy with an epitope tag,
TgMCP3ty localized primarily to the dense granules and accumulates in the PV when tagged at either termini and under the control of tubulin or rhoptry promoters. Similar observations were made for C-terminally tagged TgMCP4. Although we can not exclude that the presence of the tag interferes with targeting, this behaviour suggests that level or timing of TgMCP3 and TgMCP4 expression is important, possibly reflecting a limited accessibility of their escorter(s). Alternatively, TgMCP3 and TgMCP4 might be *bona fide* dense granule proteins but all attempts to localise the endogenous proteins remained inconclusive so far. Despite the technical hitches, the similarity between the MAR domains in these two proteins at the amino acid level points towards a similar function and therefore the subcellular localisation of TgMCP4 is likely to mimic TgMCP3.

### 4.1.3 Complex formation by MCPs

Our data demonstrates that TgMIC13 does not belong to the TgMIC1-4-6 complex. Since TgMIC13 lacks a membrane spanning domain, we reasoned that the protein is likely to be associated to a TM-MIC which would serve as an escorter and anchor TgMIC13 to the parasite surface during invasion. Concerning the architecture of the hypothetical TgMIC13 containing complex, it is interesting to note that a dozen PAN-domain containing proteins with unknown function are present in the *T. gondii* genome (Figure 4.1). PAN domains classically serve extracellular functions and indeed several of these proteins have been localized to apical secretory organelles (Chen et al, 2008). Given that the MAR domain of TgMIC1 is interacting with the PAN domains of TgMIC4 ((Saouros et al, 2005) and Prof. Stephen Matthews, unpublished), it is plausible that TgMIC13 as well as TgMCP3 and TgMCP4 are each forming complexes with homologues of TgMIC4 or with other PAN-domain containing proteins.

![Figure 4.1: Schematic of PAN-domain containing proteins encoded in the genome of *T. gondii*. Accession numbers for toxoDB are indicated to the right.](image-url)
For many lectins oligomerization was demonstrated to be important for interaction with their respective ligands. Oligomerization is a strategy to enhance the affinity and specificity of lectins toward carbohydrate ligands. For example multimerization of the major Plasmodium sporozoite surface protein, the circumsporozoite protein (PfCS), was suggested to be a requirement for sugar binding (Cerami et al, 1992b; Sinnis et al, 1994). This protein binds to heparin sulfate proteoglycans on the basolateral side of hepatocytes which is exposed to the blood stream (Cerami et al, 1992a; Frevert et al, 1993). In this way PfCS is believed to be involved in the selective attachment of sporozoites traveling in the blood circulation to these cells. Attachment is followed by invasion of hepatocytes, which initiates the liver stage development of the parasite. Structural studies have highlighted the necessity for PfEBA 175 to dimerize prior to interaction with the sialoglycoprotein glycophorin A during RBC invasion (Tolia et al, 2005). Oligomerization was also reported for a major micronemal antigen of Sarcocystis muris which is a galactose-specific lectin (Klein et al, 1998), as well as for several lectins in T. gondii, the best characterized being TgMIC2 and TgMIC3 (see introduction and (Cerede et al, 2002; Harper et al, 2004)). Analysis of recombinant TgMIC1 expressed in P. pastoris by native PAGE and gelfiltration did not reveal any sign of oligomerization for the protein on its own (data not shown). Still the protein shows binding activity in cell binding assays showing that dimerization is not an absolute requirement. Instead it seems that dimerization has been replaced by the particular stoichiometry of the TgMIC1-4-6 complex which allows for the presentation of two molecules of TgMIC1 in close proximity. The results from our invasion assays suggest that although the presentation of a single TgMIC1 is functional in invasion, this arrangement of the complex indeed allows the complex to bind to host cell receptor(s) with increased affinity which in turn results in enhanced invasion efficiency. So far we have not addressed whether TgMIC13 is able to oligomerize on its own or as part of a hypothetical complex. Identification of the components of such a complex will be necessary to resolve this issue.
4.2 Is there a molecular basis for the broad host cell range of cyst-forming coccidians?

Among the Apicomplexa the potential to invade a large variety of cell types is unique to some coccidian parasites. Similar to *T. gondii* tachyzoites, *N. caninum* tachyzoites and *S. neurona* merozoites are able to infect a large variety of cells (Ellison et al, 2001; Hemphill et al, 2006). This is probably related to their common behavior of spreading through tissues during acute infection. We found that recominant TgMIC1 and TgMIC13 are able to bind to HFFs, CHO cells and C6 rat glioma cells. Considering the potential spectrum of sialyl-oligosaccharides that can be bound by TgMIC1 and TgMIC13 as revealed by carbohydrate microarray analyses, and the wide distribution of sialic acid in vertebrate tissues, it is likely that these two lectins will bind to most cell types. Therefore, we hypothesize that TgMIC1 and TgMIC13 crucially contribute to the broad host cell range of *T. gondii*. However, among the multitude of proteins known to act on the surface of *T. gondii* others might as well play a role in this phenomenon.

TgAMA1 and TgRON proteins 2, 4, 5, and 8 form a complex at the moving junction (Straub et al, 2009). Based on the fact that the RONs were found to associate with the host cell PM (Besteiro et al, 2009), it has been proposed that the parasite provides its own anchor for invasion on the host cell surface. Furthermore it has been suggested that this could explain the broad range of cells invaded by *T. gondii* (Boothroyd & Dubremetz, 2008). Although the TgAMA1-RON2/4/5/8 complex is certainly essential for invasion of any cell type, it seems unlikely that it can provide an explanation for the considerable differences in host cell preferences and tissue tropism when comparing different apicomplexan parasites. Since the function of the complex is expected to be conserved across the phylum, it would imply that all apicomplexans have a similar host cell range, which is not true. Therefore other factors specific to subgroups of parasites should be responsible for the individual characteristics of apicomplexan parasites in discriminating between cells. How should these molecular factors look like? The domain composition of adhesive proteins is believed to reflect at least to some extent their functional characteristics. For this reason, comparing the level of conservation of protein domains across the phylum might provide indications on candidate proteins. Following these deliberations, which are the protein domains specific to coccidians? At least four different domains have to be considered: The MAR domain, the PAN domain, the chitin-binding-like (CBL) domain and the SRS domain.
Several lines of evidence support the potential role of TgMIC1 and TgMIC13, and more in general MCPs, in recognition of a broad range of cells. First, their binding characteristics are suited for engagement with a variety of cell surface sialyl-glycoconjugates which are widely distributed in vertebrate tissues. Second, a mic1-3ko strain displays decreased virulence *in vivo* (Cerede et al, 2005) and infection of mice with this attenuated strain results in reduced parasite load in a variety of tissues compared to the parental strain (Moire et al, 2009). Third, the MAR domain appears to be restricted to coccidians. Interestingly, the apple domain is specific to coccidians as well (Chen et al, 2008), although a divergent fold belonging to the PAN superfamily is present in PfAMA1 and this domain is conserved in AMA-1 homologues from all Apicomplexans. (Chesne-Seck et al, 2005; Pizarro et al, 2005)). As discussed above, it is plausible that similar to the interaction between TgMIC1 and TgMIC4, other apple domain containing proteins might associate with MCPs and form similar complexes.

*N. caninum* tachyzoites express homologues of TgMIC1 and TgMIC4, named NcMIC1 and NcMIC4. We also identified a putative TgMIC6 homologue (Sawmynaden et al, 2008); therefore we postulated the existence of a complex analogous to TgMIC1-4-6 in *Neospora*. Binding specificities of these two proteins have been characterized previously with NcMIC1 binding to sulphated GAGs (Keller et al, 2002) and NcMIC4 binding to lactose (Keller et al, 2004). We have revisited the characterization of binding preferences for NcMIC1 and found it to be specific for sialyl-oligosaccharides. These similarities to the TgMIC1-4-6 complex together with the identification of a TgMIC13 homologue in *N. caninum* corroborate the idea that these proteins fulfill 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 apple-domain containing proteins have been characterized in *Sarcocystis muris*, among them the two major micronemal antigens. These proteins were named *S. muris* lectin 1 and 2 (SmL-1, SmL-2). The lectin activity of SmL-1 depends on dimerization of the protein. Haemagglutination assays suggest binding to N-acetyl-galactosamine (Klein et al, 1998). SmL-2 and another lectin, SmL-3, show binding to galactose (Klein et al, 2003; Muller et al, 2001). Antibodies directed against the two major micronemal antigens localized at least one of the two proteins to the moving junction during host cell invasion (Entzeroth et al, 1992; Klein et al, 2003).
E. tenella is a coccidian which does not spread through tissues and does not form tissue-cysts but nevertheless also expresses at least one MCP, EtMIC3 (Labbe et al, 2005) and one apple domain containing protein, EtMIC5 (Brown et al, 2000; Brown et al, 2003). Furthermore EST and genomic data indicates that more MCPs and apple domain containing proteins might be expressed. EtMIC5 is known to form a multimeric complex with EtMIC4, a transmembrane protein composed of multiple TSP-1 and 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, our sequence and phylogenetic analyses indicate that Eimeria MCPs are divergent from those present in the cyst-forming coccidians. Therefore they might serve a different function.

4.2.2 Chitin-binding-like (CBL) domains in host cell recognition

Another domain which appears to be present only in coccidian parasites is the chitin-binding-like (CBL) domain found in TgMIC3, TgMIC8 as well as in two TgMIC8 paralogues named TgMIC8-2 and TgMIC8-3. BLAST searches identified a full set of homologues in N. caninum and indicated the presence of the CBL domain in E. tenella (Figure 4.2). Interestingly, a tryptophan residue critical for the adhesive function of TgMIC3 is conserved among all sequences (Cerede et al, 2005). In contrast, BLAST searches failed to identify a CBL domain in the genomes of Plasmodium, Theileria or Cryptosporidium. TgMIC8 clearly fulfils other functions in addition to adhesion (Cerede et al, 2005; Kessler et al, 2008). Also it has to be kept in mind that no attachment defect has been noticed in parasites depleted in TgMIC8 (Kessler et al, 2008). However, this is similar to observations on the mic1ko strain which does not exhibit any defect in parasite attachment either (Cerede et al, 2005). Consequently, the TgMIC8-MIC3 complex is another candidate potentially contributing to the capacity of T. gondii to invade such a broad range of cell types. This would be in agreement with our hypothesis since functional synergy has been demonstrated between TgMIC1 and TgMIC3 in in vivo infection (Cerede et al, 2005; Moire et al, 2009). Intriguingly, some chitin-binding domains can bind to N-acetylneuraminic acid (Raikhel-Lee et al, 1993), therefore it is possible that TgMIC8 and TgMIC3 form another sialyl-specific adhesive complex.
Figure 4.2: Alignment of CBL-domains. CBL domains were taken from TgMIC3, TgMIC8, TgMIC8.2, TgMIC8.3 and their homologues in N. caninum together with a CBL-domain identified by BLAST searches on contig 7300 of the E. tenella genome. Homologues in N. caninum were annotated in silico based on high similarity with the T. gondii proteins. In parentheses is indicated the position within the full-length sequence: TgMIC3_CBL (67-144), Tgmic8_CBL (52-126), TgMIC8-1_CBL (83-157), TGMIC8-2_CBL (108-181), NcHomTgMIC3_CBL (69-147), NcMIC8_CBL (52-127), NcMIC8-1_CBL (126-199), NcMIC8-2_CBL (87-160), EtCBL1 (contig7300).

4.2.3 Role of the surface antigen glycoprotein 1 (SAG1)-related superfamly in host cell recognition

Proteins of the SAG1-related superfamly (SRS) might also provide an explanation for the broad host cell range of T. gondii. The surface of T. gondii is populated by a family of GPI-anchored proteins of which surface antigen glycoprotein 1 (TgSAG1) is the prototypic member. The family comprises a collection of over 160 genes with stage specific expression pattern (Cleary et al, 2002; Jung et al, 2004). Among them TgSAG1 codes for the most abundantly expressed surface protein of the tachyzoite. Orthologous genes are present in the tissue-dwelling coccidians Neospora and Sarcocystis but not in more distantly related apiccomplexans (Jung et al, 2004). However, recent structural modeling studies suggested that the 6-cys-domain present in Theileria and in a family of Plasmodium gamete surface coat proteins could be related to the SRS fold (Gerloff et al, 2005; He et al, 2002; Templeton, 2007). SRS proteins were previously proposed to be involved in the primary host cell attachment and to contribute to the broad range of cell types invaded by this group of parasites. This hypothesis is based on several observations. Earlier studies using monoclonal antibodies directed against TgSAG1 and TgSAG2A showed opposite effects of inhibition and
enhancement of tachyzoite attachment to host cells respectively (Grimwood & Smith, 1992; Grimwood & Smith, 1996; Mineo et al, 1993). Potential interaction with sulfated GAGs was proposed for most SRS proteins when the crystal structure of TgSAG1 was solved (He et al, 2002). TgSAG1 forms homodimers and the two subunits build a positively charged groove conserved among SRS proteins which could serve as interaction site with negatively charged GAGs. Binding to sulfated proteoglycans was detected for recombinant TgSAG3, but not TgSAG1 (Jacquet et al, 2001). Furthermore, a strain in which the TgSAG3 gene has been disrupted revealed a decrease in adhesion of the parasite to host cells and a reduced virulence in mice (Dzierszinski et al, 2000). Conversely TgSAG1 deficient parasites showed increased host cell invasion and enhanced tissue burden in mice, but nevertheless were impaired in virulence (Rachinel et al, 2004). Taken together, these studies suggested that the different members of the SRS-family are functionally not identical.

Although these results do not exclude a role for SRS proteins in parasite attachment, their primary function appears to be related to the establishment of a chronic infection (Kim & Boothroyd, 2005; Kim et al, 2007; Saeij et al, 2008). SRS proteins expressed in the tachyzoite, the parasite form associated with acute infection, are highly immunogenic in mice and humans and TgSAG1 and TgSAG2A are known to be the immunodominant antigens (Bessieres et al, 1992; Partanen et al, 1984). TgSAG1 induces secretion of interferon (IFN)-γ by T cells, which is the major cytokine mediating resistance against Toxoplasma (Khan et al, 1988; Prigione et al, 2000; Suzuki et al, 1988). Stage conversion from the tachyzoite to the encysting bradyzoite form characteristic for chronic infection is accompanied by a switch in the expression of defined subsets of SRS-proteins (Cleary et al, 2002). Analysis of mutant parasites illigitimately expressing otherwise tachyzoite (TgSAG1) or bradyzoite (SRS9) specific proteins perturbed the establishment of a chronic infection (Kim & Boothroyd, 2005). From these experiments it was concluded that the switch of the surface coat enables the parasite to persist in face of a long-lasting immune response directed against tachyzoite specific antigens. In addition the switch is accompanied by a decrease in cytokine release thereby minimizing immunopathology to the host, an aspect that contributes to the achievement of a chronic infection by the parasite.
4.3 Benefits and intervention

Microneme proteins have been considered in the development of vaccines as well as diagnostic tests for parasitic diseases. The fact that they are secreted onto the parasite surface makes them accessible to antibodies and their important role in host cell invasion together with the discovery of invasion inhibitory antibodies indicates the possibility to block this step. For example, the micronemal antigens PfAMA1 and PfEBA175 are among the leading vaccine candidates to combat malaria. Vaccination strategies against neosporosis based on NcMIC1 alone provided partial protection in mice (Alaeddine et al, 2005) and the protection was improved when a combination of recombinant NcMIC1, NcMIC3 and NcROP2 was used for vaccination (Debache et al, 2009). This latter approach conferred complete protection from morbidity and led to a significant reduction in vertical transmission of neosporosis to pups.

The occurrence of MIC-specific antibodies in toxoplasma-positive individuals has been investigated. Antibodies specific to TgMIC1 were found in human patients and in particular, antibodies directed against the N-terminal part containing the MAR-domains were associated with acquired immunity (Holec et al, 2008). Antibody responses against TgMIC2, TgMIC3, TgMIC4, TgM2AP and TgAMA1 were detected in newborn with congenitally acquired toxoplasmosis, which is of importance for the development of reliable postnatal diagnostic tests (Buffolano et al, 2005). In a parallel study, phage display was used to identify antigenic regions in a variety of MICs. Peptide fragments from TgMIC2, TgMIC3 and TgMIC4 were found to be recognized by T cells and antibodies of adults with acquired and children with congenital infection (Beghetto et al, 2005). A subsequent vaccination study in mice was performed using these antigenic MIC-regions that resulted in generation of TgMIC2, TgMIC4, TgM2AP and TgAMA1 IgG antibodies and in a strong decrease in numbers of brain cysts following oral challenge. Another study reported that a purified TgMIC1-TgMIC4 sub-complex is a potent antigen and acts as an effective vaccine in the mouse model (Lourenco et al, 2006). TgMIC3 is as well a potent vaccine candidate and antibodies directed against the CBL domain and the EGF domains were found to confer protection in mice (Ismael et al, 2009; Ismael et al, 2003).

Another approach to develop an effective vaccine is the use of genetically attenuated parasite strains as live vaccine. Such an attenuated *T. gondii* strain (S48) is already commercialized by Intervet, Schering-Plough Animal Health, as a live vaccine (Toxovax) for animals which is administered before mating and prevents abortion over a period of two
years. However, there have been concerns about the use of this strain, since the genetic basis for the attenuated phenotype appears to be not well defined, meaning that revertants might occur and could potentially be transmitted to humans in undercooked meat. The genetic lesion of the \textit{mic1-3ko} strain with reduced virulence phenotype is well defined (Cerede et al, 2005) and was proven to be an effective vaccine against chronic and congenital toxoplasmosis in mice (Ismael et al, 2006). Vaccination almost completely prevented the generation of brain cysts after oral challenge, protected congenitally infected pups from death and again dramatically reduced the load of brain cysts in the pups. Further studies indicate that this live vaccine is also protecting from abortion in sheep (Moire et al, 2009).

4.4. Concluding remarks

In summary, our understanding of the molecular mechanisms underlying host cell invasion by apicomplexan parasites is not only of fundamental interest but also of practical relevance for the development of diagnostic tests and vaccines. Recognition of host cells through microneme proteins is an aspect that relates to parasite dissemination and disease progression. In this respect the results of this work contribute to our understanding of toxoplasma infection. The aims of the study have been partially fulfilled with the identification and characterization of two key molecular players, TgMIC1 and TgMIC13, in sialic acid-dependent host cell invasion by \textit{T. gondii}. More work is needed to elucidate the precise function of the TgMIC1-4-6 complex and TgMIC13 as well as the significance of these and other parasite ligand-host cell receptor interactions such as those mediated through PAN and CBL domains. Identification of the relevant receptor(s) carrying the sialic acid determinants used by \textit{T. gondii} to enter a host cell is the next challenging step in this study. The robust interactions of both lectins, TgMIC1 and TgMIC13, with several cell types offer a realistic chance to identify potential receptor(s) by biochemical approaches. Since substantial quantities of recombinant protein can be produced, pull-down experiments could be easily performed. Validation of potential receptor(s) would then require knockdown of the host cell receptor by RNAi or other approaches in combination with parasite invasion assays.
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