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Reference


DOI : 10.1111/j.1600-0854.2010.01148.x
PMID : 21143563
Unusual Anchor of a Motor Complex (MyoD–MLC2) to the Plasma Membrane of Toxoplasma gondii

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Toxoplasma gondii possesses 11 rather atypical myosin heavy chains. The only myosin light chain described to date is MLC1, associated with myosin A, and contributing to gliding motility. In this study, we examined the repertoire of calmodulin-like proteins in Apicomplexans, identified six putative myosin light chains and determined their subcellular localization in T. gondii and Plasmodium falciparum. MLC2, only found in coccidians, is associated with myosin D via its calmodulin (CaM)-like domain and anchored to the plasma membrane of T. gondii via its N-terminal extension. Molecular modeling suggests that the MyoD–MLC2 complex is more compact than the reported structure of Plasmodium MyoA–myosin A tail-interacting protein (MTIP) complex. Anchorage of this MLC2 to the plasma membrane is likely governed by palmitoylation.

Key words: MLC2, MyoD, myosin, pellicle, Plasmodium, Toxoplasma

Received 28 October 2010, revised and accepted for publication 7 December 2010, uncorrected manuscript published online 9 December 2010

The phylum of Apicomplexa includes several medically and veterinary relevant obligate intracellular parasites such as Plasmodium, the etiologic agent of malaria, or Toxoplasma gondii responsible for toxoplasmosis. Other members of this phylum such as Theileria, Babesia and Eimeria lead to considerable economic losses by infecting livestock.

Like most Apicomplexans, the invasive stages of T. gondii enter host cells by an active process dependent on gliding motility. Toxoplasma gondii tachyzoites exhibit three distinct modes of locomotion: circular gliding, upright twirling and helical rotation (1), and all three are powered by the parasite actomyosin system. Myosin heavy chains (MHCs) have been tailored for their implication in diverse forms of cell motility as well as in key cellular functions such as organelle transport and mitosis (2,3). These motors are typically composed of a conserved globular motor domain, a neck region associated with one or more myosin light chains and a tail region of variable length and domain composition, which interacts with the cargo and together with the motor domain, determines the specificity of myosin function. Toxoplasma gondii possesses a repertoire of 11 MHCs (TgMyoA to TgMyoK) belonging to different classes, many of them forming part of the class XIV (4). TgMyoB/C is encoded by alternative splicing of a single gene and might participate in parasite division (5,6). TgMyoA is associated with the myosin light chain MLC1 [also called myosin A tail-interacting protein (MTIP) in Plasmodium species] (7,8) and plays an essential role in parasite motility and invasion (9). This motor was previously shown to be part of a large complex composed of the gliding-associated proteins GAP45 and GAP50 (10). Two more components, GAP40 and GAP70, have recently been identified (11). This complex initially described as the ‘glideosome’ (12) is localized to the pellicle, in the space between the plasma membrane (PM) and a network of flattened cisternae called inner membrane complex (IMC) (13). GAP45 and GAP70 are acylated proteins anchored to the PM, whereas GAP40 and GAP50 are integral IMC proteins predicted to firmly anchor the motor to the IMC (14). MyoD is a motor found only in coccidians and shown to be dispensable for tachyzoites (15). As TgMyoD is more prominently expressed in bradyzoites, it may fulfill an important function in this invasive form of this chronic stage.

Typically, myosin light chains (MLCs) are calmodulin (CaM)-related proteins containing four EF-hand motifs with helix(E)-loop-helix(F) topology. The two α-helices of the EF-hand motif are positioned roughly perpendicular to one another and the ~12 amino acid loop region usually binds calcium ions (16). By binding to the IQ motif (IQxxRGGxxR) present in the neck domain of the MHC, the light chains play structural and regulatory functions and are themselves regulated by phosphorylation (17,18). TgMLC1 presents atypical EF-hands where the Ca2+-binding residues are not conserved, suggesting that Ca2+ is not required for binding to the MHC (8,19). The crystal structure of the CaM-like domain of P. falciparum MTIP with the C-terminal fragment of Plasmodium yoelii MyoA revealed a compact conformation with both domains of MTIP surrounding MyoA-tail helix (20). In this compact structure, MyoA H810 and K813...
Figure 1: Legend on next page.
are playing a major role (20). Recently, tachypleginA, a compound that modifies the N-terminal domain of MLC1, was shown to be important for host cell invasion by modulating MyoA motor activity (21).

To gain further insight into the composition and role of myosin motors in Apicomplexans, and to identify putative MLCs, we combined database mining of the apicomplexan parasite genomes with detailed phylogenetic analysis of the genes coding for CaM-like proteins. Moreover, we determined the subcellular localization of six new putative MLCs in *T. gondii* and two in *P. falciparum*. We then focused on TgMLC2 and proved that it is associated with TgMyoD. A detailed investigation revealed that the N-terminal extension of MLC2 is responsible for the localization of TgMyoD to the pellicle and anchoring to the PM.

**Results**

*Toxoplasma gondii* possesses a repertoire of seven putative myosin light chains

A database search across the apicomplexan genomes was performed with the objective to find the EF-hand-containing protein sequences closely related to CaM (an alignment of these sequences can be found in dataset Appendix S1). The subsequent phylogenetic analysis showed a clear clustering of the EF-hand-containing proteins of apicomplexan parasites into five groups (Figure S1 and Table S1). The most highly conserved of these clusters comprises the ‘true’ CaMs for which one copy is found in each organism’s genome. The short branch lengths indicate the high sequence conservation with the respective sequences of *P. falciparum* and *Basbesia bovis* still sharing 123 of 149 (82%) identical amino acids. The putative apicomplexan MLCs form a well-supported cluster with copies of MLC1 (MTIP), MLC4 and MLC7 present in the major apicomplexan lineages (MLC4 being absent only from *Cryptosporidium*) (Figure 1A). In addition, the coccidian parasites have another four putative MLC sequences (MLC2, MLC3, MLC5, MLC6) that appear to have originated by relatively late gene duplication events within this lineage, with MLC6 being the most divergent putative MLC. TgMLC6 (EEA98182) corresponds to TgCaM2, previously reported to be located at the conoid (22).

The alignment of the *T. gondii* MLCs shows that they all contain four ‘degenerated’ EF-hands because they lack the residues involved in the binding of the calcium ion, which compose the CaM-like domain (Figure 1B). As previously described for MLC1 (8), the last EF-hand motif is highly degenerated suggesting that the binding to MHC-degenerated IQ motif could be Ca$^{2+}$-independent. More surprisingly, all MLCs, except TgMLC5, exhibit an N-terminal extension compared to TgCaM (Figure 1B). These N-terminal extensions do not exhibit any particular motif or domain except for the extension of TgMLC3, where a coiled-coil domain is predicted (25).

A gene coding for a protein referring to myosin regulatory light chain (MRLC) in *Toxoplasma* genome (ToxoDB) is present and conserved in all Apicomplexans analyzed here. This group being phylogenetically related to myosin light chain, we decided to call its members, putative myosin light chain 7, MLC7 (Figure 1A,C). MLC7s present conserved N-terminal and C-terminal ends of each of them containing one EF-hand domain. Table S2 records the degree of homology between the MLCs of different Apicomplexans and the evidence for their expression based on detection of specific expressed sequence tags (ESTs) or peptides by mass spectrometry (MS). In this study, all the genes coding for the putative MLCs in *T. gondii* tachyzoite and *P. falciparum* D10 blood stages have been experimentally annotated, confirming that these genes are all transcribed in these stages.

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**Figure 1:** Repertoire of myosin light chain-like proteins in *Apicomplexa*. A) Phylogenetic tree of apicomplexan myosin light chain-like sequences. This tree is part of the full phylogenetic maximum likelihood tree of apicomplexan myosin light chain, calmodulin, and centrin related EF-hand-containing sequences presented in Figure S1 and highlights the myosin light chain-like sequences found in *Apicomplexa*. The tree topology shown is the best (ln likelihood $= -18 472.93$) of 10 trees generated with PHYLP’S PROML using randomized sequence input orders. Bootstrap support values above 50% (of 100 replicates) from maximum likelihood (ML) and neighbor-joining (NJ; PROTDIST and NEIGHBOR) analyses are indicated above and below branches, respectively. The full species names are as follows: *B. bovis, Babesia bovis; C. hominis, Cryptosporidium hominis; C. muris, Cryptosporidium muris; C. parvum, Cryptosporidium parvum; E. tenella, Eimeria tenella; N. caninum, Neospora caninum; P. berghei, Plasmodium berghei; P. falciparum, Plasmodium falciparum; P. knowlesi, Plasmodium knowlesi; P. vivax, Plasmodium vivax; P. yoelii, Plasmodium yoelii; T. annulata, Theileria annulata; T. gondii, Toxoplasma gondii; T. parva, Theileria parva. Database accession numbers are indicated in the figure behind the species names, ToxoDB for *T. gondii*, PlasmodDB for *Plasmodium* species, assemble contigs for *N. caninum*, Genbank for *Cryptosporidium* species, *B. bovis* and *T. annulata*. B) Multiple alignment of the putative *T. gondii* MLCs performed using MULALIGN (http://www.bioinf.genotoul.fr/multalin/) (23). Identical residues are in red and residues conserved in more than 50% but less than 90% are in green. The N-terminal extension is indicated by a purple line, the four degenerated EF-hands by blue lines. Genbank accession numbers: TgMLC1 (AY048862), TgMLC2 (EF175162), TgMLC3 (EF175163), TgMLC4 (DQ983321), TgMLC5 (DQ983322). C) Multiple alignment of the putative apicomplexan MLC7s performed using CLUSTAL W (http://www.ebi.ac.uk/Tools/clustalw2/index.html) (24). Identical residues are in red, strongly similar residues are in blue and weakly similar residues are in green. The asterisks highlight the residues usually involved in the co-ordination of Ca$^{2+}$ in a canonical EF-hand motif (18). The coiled-coil domain prediction was performed using COILS (http://www.ch.embnet.org/software/COILS_form.html) (25) and is indicated in yellow. Accession numbers: TgMLC7 (Genbank EEA97615), NcMLC7 (N. caninum assemble contigs, contig_959), EfMLC7 (Genbank ELMER_contig_00029736), PfMLC7 (Genbank XP_001347585), PnMLC7 (Genbank EDL2438), PyMLC7 (Genbank EAA16665), PfMLC7 (Genbank CAQ38989), BbMLC7 (Genbank XP_001611994), TaMLC7 (Genbank XP_0630404) and CmMLC7 (Genbank EEA08050).
**Subcellular distribution of the putative MLCs in *T. gondii* and *P. falciparum***

To determine the localization of the different MLCs in *T. gondii*, we generated transgenic parasites stably expressing the transgene with an epitope tag inserted either at the C-terminus (Ty-1) or at the N-terminus (Myc) of the protein under the control of the tubulin promoter (Figure S2A). We confirmed by immunoblot analysis using anti-Ty or anti-Myc antibodies that the tagged proteins migrate as a single band of the expected size (Figure 2A). TgMLC2Ty is distributed at the parasite periphery as previously reported for TgMLC1 (TgMLC1Ty shown as control) (Figure 2B). In contrast, TgMLC3 and TgMLC7 are cytosolic, TgMLC4 is localized around the nucleus, apparently associated with vesicles at the proximity of the endoplasmic reticulum, whereas TgMLC5 is present both in the conoid and in the nucleus but excluded from the nucleoli (Figure 2B). The same localizations were observed for all the MLCs when the tag was inserted at the other extremity (Figure S2B).

In *P. falciparum*, two putative MLCs orthologous to TgMLC4 and TgMLC7 were identified and named PfMLC4 and PfMLC7. To study their localization in *P. falciparum*, we generated parasites expressing episomal PfMLC4 and PfMLC7 fused to an N-terminal hemagglutinin (HA) tag under the control of the *crt* promoter. There is no information regarding the expression profile of PFF1320c (PfMLC4) throughout the intraerythrocytic cycle, whereas the expression profile of PF10_0301 (PfMLC7) is similar.

**Figure 2: Localization of putative myosin light chains in *T. gondii***. A) Total lysates from wild-type parasites (RH) and parasites stably expressing TgMLC1Ty, TgMLC2Ty, TgMLC3Ty, MycTgMLC4, MycTgMLC5 and MycTgMLC7 were analyzed by immunoblot using anti-Ty (left panel) or anti-Myc antibodies (right panel), respectively. The catalase (CAT) was used as a control. Arrows indicate the proteins of interest. B) The subcellular localization of the TgMLCs in intracellular tachyzoites was determined by IFA using anti-Ty or anti-Myc antibodies. Anti-GAP45 antibodies stained the periphery of the parasites. Arrows point to the conoid. Scale bar: 2 μm.
MyoD–MLC2 is Anchored to the Plasma Membrane of Toxoplasma

Figure 3: Localization of putative myosin light chains in P. falciparum. A) Immunoblot analysis of wild-type parasites (D10) and transgenic parasites expressing HA-PfMLC4 using anti-HA antibodies. Actin was used as a loading control. HRBC, human red blood cells. Arrow indicates the protein of interest. B) Localization of HA-PfMLC4 and HA-PfMLC7 on live transgenic parasites using anti-HA antibodies. DAPI was used to visualize the nuclei. Scale bar: 2 μm.

to crt. The expression of the transgenic HA-PfMLC4 was confirmed by immunoblot analysis using anti-HA antibodies with a band at the expected size (18 kDa). However, HA-PfMLC7 remained undetectable (Figure 3A). PfMLC4 localizes in the cytosol of the transfected parasites, whereas TgMLC4 seems to be associated with the ER (Figure 2B). No colocalization was obtained with anti-ERD2 antibodies (data not shown). These data suggest that the proteins act independently and serve different functions. When expressed as an N-terminal HA-tagged copy under the control of crt promoter, PfMLC7 showed a cytosolic punctate localization (Figure 3B).

The N-terminal extension of TgMLC2 targets the protein to the plasma membrane

The distribution of TgMLC2 at the periphery of the parasite prompted us to focus on the characterization of this putative MLC. The localization was confirmed by the generation of specific antibodies against TgMLC2 and by the insertion of a C-terminal epitope tag in the endogenous locus by single crossing over, which is favored in the ku80-ko background strain (26,27). The knock-in (KI) approach eliminates potential artifacts caused by overexpression or inaccurate timing of expression of second copy of the transgene. However, it only works if the insertion of the tag does not interfere with the function of the protein (or if the gene is not essential in that stage). The tagging of MLC2 in the endogenous locus was confirmed by genomic polymerase chain reaction (PCR) (data not shown) and by immunoblot analysis (Figure 4A). TgMLC2Ty-KI localized at the parasite periphery of intra-cellular parasites (Figure 4B). Specifically, the anti-MLC2 antibodies (raised against an Escherichia coli produced recombinant fragment corresponding to the N-terminal extension of TgMLC2) recognized a band of ca. 45 kDa molecular weight in wild-type RH. A slightly larger product was detected in the ku80-ko modified in the TgMLC2 locus by the insertion of three Ty-tag epitopes at the C-terminus of MLC2 (MLC2Ty-KI). By immunofluorescence assay (IFA), anti-Ty antibodies labeled the periphery of the parasites, confirming that TgMLC2 is present at the pellicle of the tachyzoites (Figure 4B).

TgMLC2, like TgMLC1, exhibits an N-terminal extension compared to CaM, as shown in the alignment of the putative TgMLCs (Figure 1B). To determine the role and importance of this region along with the C-terminal CaM-like domain, two green fluorescent protein (GFP)-Ty fusions were generated with either the N-terminal extension (aa 1 to 230) or the C-terminal domain containing the EF-hands (aa 226 to 397) (Figure S2). The localization of these two chimeras was compared with the full-length version of TgMLC2 (Figure 4D). On immunoblots, anti-Ty antibodies detected bands at positions corresponding to the expected sizes of 42 kDa for TgMLC2, 53 kDa for the NtTgMLC2GFPTy and 44 kDa for the CtTgMLC2-GFP (Figure 4C). NtTgMLC2GFPTy, like TgMLC2, colocalizes with GAP45 at the parasite periphery. In contrast, CtTgMLC2GFPTy remains cytosolic (Figure 4D), indicating that the N-terminal extension is necessary and sufficient to target TgMLC2 to the pellicle.

To distinguish if TgMLC2 is associated with the IMC or the PM, we performed IFA on parasites treated with Aeromonas hydrophila aerolysin. As described with α-toxin (28), this toxin binds to glycosyl-phosphatidylinositol (GPI)-anchored proteins creating pores, which causes the PM to swell away from the IMC. Under this condition, the distribution of the GPI-anchored major surface antigen 1 (SAG1) and the IMC gliding-associated protein GAP45 is clearly distinct (10). TgMLC2Ty-KI as well as NtTgMLC2GFPTy colocalized with SAG1 indicative of an anchoring to the...
Figure 4: The N-terminal domain of TgMLC2 is responsible for its localization to the plasma membrane. A) Immunoblot analysis of wild-type parasites (RH) and parasites expressing the endogenously tagged TgMLC2Ty-KI using anti-Ty and anti-MLC2 antibodies, respectively. The CAT was used as a loading control. Arrows indicate the proteins of interest. B) IFA showing the localization of TgMLC2Ty at the periphery of the parasites using anti-Ty and anti-MLC2 antibodies. C) Immunoblot analysis of parasites stably expressing TgMLC2Ty (full), NtTgMLC2GFPTy (Nt) and CtTgMLC2GFPTy (Ct) using anti-Ty antibodies. Arrows indicate the proteins of interest. D) The subcellular localization of the full, Nt and Ct-MLC2 constructs was determined in intracellular tachyzoites using anti-Ty antibodies. The periphery of the parasites was visualized by TgGAP45 staining. Scale bar: 2 μm. E) The distribution of TgMLC2Ty-KI and NtTgMLC2GFPTy at the pellicle was determined in extracellular tachyzoites treated with aerolysin using anti-Ty antibody. The PM and the IMC were visualized by TgSAG1 and TgGAP45 staining, respectively. Arrows point the IMC and arrowheads indicate the PM. Scale bar: 2 μm.
PM (Figure 4E). These results contrast with TgMLC1, which segregates with the IMC together with the other members of the motor complex MyoA–GAP45 (10,11).

**TgMLC2 is the light chain of TgMyoD**

Given its localization to the PM, TgMLC2 is unlikely to serve as a light chain for TgMyoA. To identify the MHC interacting with MLC2, co-immunoprecipitation (co-IP) experiments using anti-Ty antibodies were performed on stable parasite lines expressing TgMLC2 or NtMLC2- or CtMLC2-GFP fusions. Extraction of parasite protein complexes and co-IP were executed in the presence of Triton-X-100 and the eluted proteins were analyzed by Coomassie blue-stained SDS–PAGE. As shown in Figure 5A, few proteins were present. The high molecular weight band around 90–95 kDa (band 1) commonly bound to TgMLC2 and CtTgMLC2GFP-Ty was identified as TgMyoD by MS (seven peptides, coverage 15%; Table S3). The band 2 corresponds to the full-length TgMLC2-Ty. These results were confirmed by immunoblot analysis using anti-MyoD and anti-Ty antibodies (Figure 5B). The antibodies directed against a peptide in the N-terminus of MyoD (15) reacted with a single 90-kDa band in the co-IP with TgMLC2 and the CaM-like domain samples, but not in the sample resulting from the co-IP with the Nt domain (Figure 5B, right panel). The successful immunoprecipitation of TgMLC2-Ty, NtMLC2GFP-Ty and CtMLC2GFP-Ty was confirmed by Western blot with anti-Ty antibodies (Figure 5B, left panel). These data show that the EF-hands are involved in the interaction of TgMLC2 with TgMyoD. Specific antibodies raised against the N-terminal extension of TgMLC2 were used to investigate the myod-ko parasites (15). Interestingly, and in contrast to wild-type parasites, no TgMLC2 is detectable in the absence of TgMyoD (Figure 5C). The same phenomenon was previously observed with the related motors TgMyoA and TgMLC1 (Meissner and Soldati, unpublished), indicating that the light chains are only stable in the presence of their corresponding heavy chain. The complete disappearance of TgMLC2 strongly suggests that TgMyoD is the only motor associated with TgMLC2.

**Structure of TgMLC2–TgMyoD-tail complex**

We used the PfMTIP–MyoA crystal structure (20) as a template for homology modeling of the complex formed by TgMLC2 lacking the long N-terminal extension with the TgMyoD-tail region (Figure 6A). For simplicity, we will refer to the two proteins as MLC2 and MyoD for the remainder of the text. The MLC2 model is predicted to consist of two subdomains. The N-domain formed by residues 228 to 292 contains EF-hand motifs 1 and 2, whereas the C-domain formed by residues 299 to 367 contains EF-hand motifs 3 and 4. In analogy to the PfMTIP structure (20), the subdomains are predicted to be connected by a short unstructured ‘hinge’ loop formed by residues 293 to 298 (Figure 6A). MyoD-tail residues 803 to 816 containing a degenerate IQ motif (ICANVRKRLVQ) are modeled as an α-helix.

The resulting model of the MLC2–MyoD complex shows that the two MLC2 subdomains surround the MyoD-tail helix in a clamp-like manner (Figure 6B). The MLC2–MyoD interface contains predominantly non-polar residues (55%). Charged and polar neutral amino acids contribute for 28 and 17%, respectively. Similar to the experimentally solved structures, the N-domain is predicted to form crucial interactions with the tail region (20,29). In contrast, the interactions involving the C-domain appear more dominant than in PfMTIP–MyoA crystal structure (20). Previous studies have shown that a glycine or other small residue is not required at the seventh position of the IQ motif to accommodate MLC binding (20). In fact, the same group showed through mutagenesis and structural studies that mutation of
Figure 6: Structural overview of the TgMyoD-tail bound to the CaM-like domain of TgMLC2. A) Sequence alignment of the C-terminal part of PyMyoA, TgMyoA and TgMyoD and of the CaM-like domain of PfMTIP, TgMLC1 and TgMLC2. The positions of the secondary structure elements (helices represented as springs and strands represented as arrows) are based on the PyMyoA–PfMTIP structure solved by X-ray (20). B) Cartoon representation of TgMyoD-tail bound to TgMLC2, view along the MyoD-tail helix axis in white. The two first EF-hands (N-domain) are colored in green and the two last ones (C-domain) are colored in blue. C) Hydrophilic network around the MyoD-tail shown as stick representation in white. The contacting residues of TgMLC2 are colored according to their location, C-domain residues are in blue, hinge region residues in orange and N-domain residues in green and distances are shown in Å. D) Illustration of hydrophobic interactions between the N-terminal extremity of MyoD-tail and C-domain of TgMLC2. Non-polar residues of the C-domain of TgMLC2 are shown as stick representation in violet, and MyoD-tail buried non-polar residues are shown as sticks in yellow. E) Superimposition of TgMyoD-tail helix in the TgMLC2–MyoD complex, in white, with PfMyoA-tail helix of the PfMTIP–MyoA complex, in cyan.
G812K in PfMyoA promotes a compact conformation of the complex. In our TgMyoD model, R812 occupies the seventh position of the IQ motif forming a tight network of hydrogen bonds with residues in the C-domain (Figure 6C). The tighter interactions with the C-domain may contribute to the compactness of the interface. The predicted accessible surface area in the interface of the MyoD–MLC2 complex corresponds to 884 Å², compared to 1108 Å² for the homology-modeled TgMLC1–MyoA complex and 958 Å² for the PfMTIP–MyoA complex (20).

Residues F305, F306, F309 and F342 from the C-domain of MLC2 form a hydrophobic cluster that interacts with residues A803, I806, C807, A808 and V810 in the MyoD-tail helix (Figure 6D). The presence of C807, which replaces the conserved glutamine in the MyoD IQ motif, favors the interaction with the hydrophobic cluster in the C-domain of MLC2. Glutamine, being a polar residue, cannot be accommodated in this position. N809 of MyoD-tail is one of the key residues in the interface with MLC2. Mostly, histidine residues are preferred in this position and H811 as well as H812 are the corresponding residues in TgMyoA and PfMyoA. The PfMyoA and TgMyoA tail regions differ in MyoD positions 804 and 807 that are involved in the interactions with MLC2 (Figure 6E). Conserved basic residues R811 and R812 in the MyoD-tail helix are involved in a tight network of polar interactions with MLC2 residues E331, E332, M361 and L362, E364. R805 forms hydrogen bonds with H294 and D297 in the MLC2 hinge region (Figure 6C). Thus, most residues in the MLC2 C-domain are predicted to be involved in direct interaction with the MyoD-tail.

No accessory protein was found to anchor TgMLC2–MyoD to the plasma membrane

A comparison of the co-IPs with TgMLC2Ty and TgMLC1Ty by Western blot revealed that neither TgMyoA nor TgGAP45 is interacting with the TgMyoD–MLC2 (Figure 7A). It has been recently reported that tachypleginA, a small-molecule inhibitor of T. gondii motility, prevents TgMyoA-GAP45-GAP50-GAP40 (11), whereas TgMLC2 precipitates TgMyoD only (Figure 7B). As none of these two proteins have transmembrane domains, an *in silico* investigation of potential acylation sites was undertaken. Both TgMLC2 and TgMyoD are predicted to be palmitoylated on several cysteines (Figure S3A) with good scores (30). Fractionations performed on the TgMLC2 Ty-KI strain showed that the MLC is partially solubilized in PBS, carbonates and salt, which likely corresponds to a cytosolic pool also observed by IFA (Figure 4B). In contrast, the totality of the protein can only be fully solubilized in the presence of detergent (Figure 7C), strongly suggesting that its interaction with the membrane is due to lipid modification. Taken together, these results provided further evidence that TgMyoD–MLC2 is a two-component complex.

Discussion

The repertoire of EF-hand-containing proteins obtained from database mining of the available apicomplexan genomes were subjected to a phylogenetic analysis to identify novel putative myosin light chains. The next objective was to determine their subcellular localization and assign them experimentally to the previously described MHCs (4) and ultimately to unravel their biological function. The number of putative MLCs identified in this study is inferior to the number of MHCs, suggesting that some MLCs are likely associated with several motors. Alternatively, CaM and CaM-like proteins might also act as MLCs, as frequently observed for the unconventional myosins (31). In this study, we validated the phylogenetic analysis by confirming that MLC2 is indeed an MLC and we identified the class XIV myosin TgMyoD as the unique motor associated with MLC2 by co-IP and MS analysis.

GFP fusions of the two distinct domains of MLC2 showed that its C-terminal CaM-like domain is necessary and sufficient to interact with TgMyoD, whereas its N-terminal extension is essential for the localization of the motor to the parasite pellicle. Following treatment with the aerolysin toxin, which separates the PM from the IMC, we could establish that the MyoD–MLC2 motor is anchored to the PM. Only few myosins have been shown to interact directly with membranes. The tails of myosins I and V contain a pleckstrin homology (PH) domain, possibly involved in phospholipids binding, while additional basic residues also contribute with important non-specific electrostatic interactions (32,33). Alternatively, the myosin I in *Dictyostelium discoideum* has recently been reported to be anchored to the membrane via C-terminal farnesylation (34). In the present study, we show that MLC2 is substituting for the function of the tail by bringing the motor to its site of action. Metabolic labeling and co-IP experiments suggested the absence of additional protein in this pellicle complex (Figure 7B). The simple composition of the complex (MyoD–MLC2) as well as the fractionation experiments strongly suggest that MLC2 itself is directly anchored into the PM. Consistent with this interpretation, MLC2 is predicted to possess three putative palmitoylation sites in its N-terminal extension (type III sites C17, C117 and C119; Figure S3A). The potential sites of palmitoylation on TgMyoD could enhance the interaction with the membrane once the motor is in close proximity with the lipid bilayer via its interaction with MLC2.

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Figure 7: No accessory proteins were found in TgMLC2–TgMyoD complex. A) Extracellular parasites stably expressing TgMLC1Ty and TgMLC2Ty were subjected to immunoprecipitation with the anti-Ty antibodies. Co-IPs were analyzed by Western blot using anti-Ty (left panel), anti-TgMyoA (middle left panel), anti-TgGAP45 (middle right panel) and anti-TgMyoD (right panel) antibodies. Arrows indicate the proteins of interest and asterisks indicate the IgG heavy and light chains that are cross-reacting with the secondary antibody used. B) [35S]-labeled methionine/cysteine parasites stably expressing MLC1Ty and MLC2Ty were subjected to co-IP with anti-Ty antibodies. Eluted proteins were visualized by autoradiography and their identification is indicated on the right panel. C) The solubility of endogenous TgMLC2Ty-KI was studied by fractionation after extraction in PBS, PBS/NaCl, PBS/Na2CO3 or PBS/TX100. Its distribution in the different fractions was assessed by Western blot using anti-Ty antibodies. MLC1 and CAT were used as fractionation controls.

We treated parasites with tachypleginA and showed that TgMLC2 is not modified by this drug (Figure S3). These data are consistent with the fact that the modified peptide identified on MLC1 is absent on MLC2 (21).

TgGAP50 is described as the receptor for TgMyoA to the IMC (10) and was recently shown to be responsible for the immobilization of the MyoA–MLC1 motor in the matrix membrane of the IMC (14). In sharp contrast, MyoD–MLC2 is not firmly anchored to the IMC but instead fluidly associated with the PM. This dramatic difference in localization and nature of anchoring is likely to reflect fundamentally distinct motor function.

TgMyoD is the smallest T. gondii MHC and has the closest relative to TgMyoA, it exhibits the same kinetic characteristics as TgMyoA (15), classic fast type myosin. A conventional homologous recombination strategy was used to create a mutant lacking TgMyoD in the virulent RH strain showing TgMyoD is not necessary for survival of the tachyzoite stage (15). However, MyoD is more abundantly expressed in bradyzoites and it is plausible that this motor fulfils a more prominent role in the chronic stage (15). Interestingly, TgMLC2 completely disappears in the myod-ko parasites. A dramatic drop of TgMLC1 was already observed in TgMyoA-inducible knockout (9), suggesting that the interaction between light chain and heavy chain contributes to the stability of each component of the motor.

Homology modeling predicts that the MLC2 forms a tight clamp around the MyoD-tail helix and adopts a more closed and compact conformation than observed in the structures of other MLC–MHC complexes. Special features in both the light and heavy chains are contributing to this compactness and the uniqueness of the MLC2–MyoD interface. The unique composition and the tight MLC2–MyoD interface make it an attractive target for the development...
of anti-cocccidial drugs. Small molecule compounds, which are designed to inhibit a specific protein–protein interactions, are expected to diminish the likelihood of acquired drug resistance by the parasite through permissible complementary mutations of interacting residues.

Materials and Methods

Multiple protein sequence alignments and phylogenetic analysis

Publicly available protein and EST sequences were taken from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/), Toxoplasma and Plasmodium genomic resources (http://www.toxodb.org and http://www.plasmodb.org, respectively) and the Wellcome Trust Sanger Institute (http://www.sanger.ac.uk/). After randomizing sequence order, multiple sequence alignments were created with ClustalW v1.83 (35) using the default pairwise and multiple alignment parameters. Phylogenetic maximum likelihood and neighbor-joining analyses were carried out using the PHYLIP programs SEQBOOT, PROML, PROTDIST, NEIGHBOR and CONSENSE (version 3.69) (36). The Jones-Taylor-Thornton model was selected in PROML and PROTDIST, global rearrangements were employed in PROML and sequence input order was randomized in PROML and NEIGHBOR. The resulting trees were visualized with TREEVIEW (37). All the accession numbers of the centrin, CaM, CaM-like, MLC and MLC-like sequences used in the phylogenetic analysis are listed in Table S1 and the sequence alignment performed for the phylogeny is listed in Appendix S1.

Sequence analysis

Sequences were analyzed using MULTalin (http://www.bioinf.cea.fr/multalin/) (23) and ClustalW v1.83 (http://www.ebi.ac.uk/clustalw/) (24) programs. The presence of canonical EF-hand in the MLCs was assessed using SMART (http://www.smart.embl-heidelberg.de) (38,39). Coiled-coil regions were predicted using COILS (http://www.ch.embnet.org/software/COILS_form.html) (25) and potential palmitoylation sites were predicted using CSS/PALM 2.0 (http://www.csspalm.biocuckoo.org) (30).

Toxoplasma gondii and Plasmodium falciparum cultures

Toxoplasma gondii tachyzoites [RHhxgprt strain, ΔKu80hxgprt strain (27) and its derivatives expressing the epitope-tagged proteins] were grown in confluent human foreskin fibroblasts (HFFs) or in Vero cells maintained in DMEM (GIBCO, Invitrogen) supplemented with 5% fetal calf serum, 2 mM glutamine and 5% calf serum, 2 mM glutamine and 25 μg/mL gentamicin.

Plasmodium falciparum D10 strain was grown in A+ erythrocytes in RPMI-1640 medium with glutamine (Life Technologies), 0.2% glucose, 0.2% sodium bicarbonate, 25 μM HEPES, 5% human serum and 0.1% AlbuMax II (Life Technologies). Parasites were synchronized by two consecutive sorbitol treatments as previously described (40).

Cloning of DNA constructs

Total RNAs were isolated from Toxoplasma tachyzoites (RH strain) and from mixed P. falciparum D10 strain using Trizol (Invitrogen). Total cDNAs were then generated by reverse transcription-polymerase chain reaction (RT-PCR) performed with the Superscript II reverse transcriptase (Invitrogen) according to the manufacturer’s instructions. All amplifications were performed with the LaTaq polymerase (Takara) and the primers listed in Table S2. All TgMLC open reading frames (ORFs), except TgMLC6, were annotated after amplification from cDNA using primers upstream the ATG and downstream the stop codon and cloned into the pGEM T-easy vector (Promega). The full-length coding sequences were then amplified and cloned into the pTUB8MycGFPPMyoAtailTy-HX (8) between EcoRI and NsiI restriction sites or between NsiI and PacI sites to generate pTUB8TgMLC1Ty, pTUB8TgMLC2Ty, pTUB8TgMLC3Ty, pTUB8MycHisTgMLC4, pTUB8MycTgMLC5 and pTUB8MycHisTgMLRC.

To study the determinant of TgMLC2 pellicle localization, sequences encoding the N-terminal extension (aa 1 to 230) or the C-terminal part (aa 226 to 367) were amplified and cloned into a modified pTUB8MycGFPPMyoAtaiTy-HX between the EcoRi and NsiI restriction sites in frame with the GFP and the Ty-tag to create pTUB8NtTgMLC2 GFTPv-HX and pTUB8CtTgMLC2GFTPv-HX, respectively.

For TgMLC2 KI, TgMLC2 genomic sequence was obtained from ToxoDB database. Primers were designed to amplify 1374 bp of the 3’ end of the gene that contains a unique XhoI restriction site. The PCR fragment was then cloned into pTUB8MycGFPPMyoAtaiTy-HX (8) between XpnI and NsiI sites.

The full-length PIFLC4 and PIFLC7 genes were amplified from P. falciparum D10 strain using primers PIFLC4-1 and PIFLC4-2 for PIFLC4 or PIFLC7-1 and PIFLC7-2 for PIFLC7. Primers were designed to amplify the gene as predicted (PIFLC4 accession XP_0966255, PIFLC7 accession number XP_001347588) and as annotated (PFF1320c and PF100_301 for PIFLC4 and PIFLC7, respectively) in Plasmodb (http://www.plasmodb.org/).

Coding sequences were then cloned into a modified pARL-GFP plasmid (kind gift from Brendan Crabb and Alan Cowman, WEHI) downstream the N-terminal HA tag.

Parasite transfections and selection of stable lines

Transfections of T. gondii were performed by electroporation as previously described (41). The hypoxanthine-xanthine-guanine phosphoribosyl transferase (hxgprt) gene was used as a positive selectable marker in the presence of mycophenolic acid (MPA, 25 mg/mL) and xanthine (50 mg/mL), as described before (42). TgMLC2Ty KI transfection was performed in the ΔKu80hxgprt strain using 20 μg of vector linearized by XhoI as described previously (27) and MPAXanithine selection.

Transfections of P. falciparum clone D10 were carried out by electroporation using 100 μg of plasmid. Drug selection was performed using 5 μM WR99210 for the human dhfr-based plasmid PRL1 as previously described (43,44).

Production of specific polyclonal antibodies against TgMLC2

For the production of TgMLC2-specific polyclonal antibodies, primers pair TgMLC2-9f/TgMLC2-10 was used to amplify the N-terminal part of TgMLC2 sequence encoding for aa 1 to 230. The fragment was then cloned between the Ncol and SacI restriction sites of the bacterial expression plasmid pET6H in frame with six histidines (kindly provided by A. Houdusse, Paris). The recombinant protein was expressed in E. coli BL21 strain and purified by affinity chromatography on nickel-nitriotic acid (Ni-NTA) agarose (Qiagen) according to the manufacturer’s protocol under denaturing conditions. The purified recombinant protein was then used to immunize two rabbits (Eurogentec S.A.) according to their standard protocol.

IFA and confocal microscopy

Parasite-infected HFF cells were fixed with 4% paraformaldehyde or 4% paraformaldehyde−0.05% glutaraldehyde in PBS for 15 min, followed by 5 min of neutralization with 0.1 M glycine in PBS. Fixed cells were permeabilized with 0.2% Triton-X-100 in PBS (PBS/TX) for 20 min and blocked with 2% BSA in PBS/TX for 20 min. The cells were then incubated for 1 h with primary antibodies (anti-Myo, anti-Ty 1:1000, anti-GAP43 1:5000) followed by goat-anti-rabbit or goat-anti-mouse immunoglobulin G (IgG) conjugated to Alexa Fluor 488 or Alexa Fluor 594 (Molecular Probes, Invitrogen). Finally, glass coverslips were incubated for 10 min.
Plasmodium falciparum immunofluorescence assays were performed on 4% paraformaldehyde–0.0075% glutaraldehyde fixed parasites as previously described (45). Parasites were permeabilized for 10 min in PBS-Triton 0.1% and blocked in 3% BSA/PBS for 1 h. Mouse anti-HA (diluted 1:500; Covance) was added and allowed to bind for 1 h in 3% BSA/PBS. Primary antibodies were consequently detected by incubation for 45 min with Alexa Fluor 488-conjugated anti-mouse IgG (1:3000; Molecular Probes) containing 1:1000 DAPI.

Confocal images were collected with a Leica laser scanning confocal microscope (TCS-NT DM) and SP2) using a 1000 Plan-Apo objective with numerical aperture (NA) 1.4. Single optical sections were recorded with an optimal pinhole of 1.0 (according to Leica instructions) and 16 times averaging. Stacks of sections were recorded at ~0.2-μm vertical steps and projected using the maximum projection tool.

Aeromonas hydrophila aerolysin experiments

Prior to use, purified recombinant aerolysin was activated for 15–20 min at 37°C in PBS with 2 μg of trypsin diluted at 1 mg/ml in hepes buffer saline (HBS) (140 mM NaCl, 2.7 mM KCl, 20 mM HEPES, pH 7.4). Freshly harvested parasites were washed in PBS and incubated for 10 min at 37°C on coverslips coated with poly-lysine (1 mg/mL). Parasites were then treated with aerolysin (80–100 ng/ml) for 3 h at 37°C before fixation for 10 min in 4% paraformaldehyde or 4% paraformaldehyde-0.005% glutaraldehyde. Immunofluorescence assays were then performed as described earlier with anti-Ty and anti-GAP43 antibodies.

Immunoblotting

Freshly released tachyzoites were harvested, washed in PBS, resuspended and boiled directly into SDS sample buffer. Polyclaylamide gels were run under reducing conditions in the presence of 1 μl dithiotreitol (DTT) in the sample buffer. Proteins in the gel were transferred onto a nitrocellulose membrane using a semi-dry electrobolter, and were incubated for 1 h with respective mouse monoclonal antibodies or rabbit polyclonal antisera in PBS, 0.05% Tween-20 and 5% non-fat milk powder. After washing, the nitrocellulose membrane was incubated for 45 min with a peroxidase-conjugated goat anti-mouse or anti-rabbit antibody (Biorad) and bound antibodies visualized using the ECL plus system (GE Healthcare Life Sciences).

Mixed iRBCs expressing HA-PLMCL4 and HA-PLMCL7 were treated for 5 min with 0.15% saponine in PBS on ice to lyse the red blood cells. Total parasitic proteins were then extracted in reducing Laemmli sample buffer, allowed one missed cleavage with the trypsin enzyme selected, one minute for 10 seconds) and

Subcellular fractionations

Freshly released tachyzoites were harvested, washed in PBS and then resuspended into PBS, PBS and 1% Triton-X-100, PBS and 1 μ NaCl, or PBS and 0.1% Na2CO3, pH 11.5. Parasites were lysed by freezing and thawing followed by sonication on ice. Pellet and soluble fractions were separated by centrifugation at 14,000 rpm for 1 h at 4°C.

Co-immunoprecipitation

Freshly released tachyzoites were harvested, washed in PBS and frozen. The parasites were resuspended in Co-IP buffer [1% Triton-X-100, 50 mM Tris–HCl, pH 8, 150 mM NaCl, 5 mM ethylenediaminetetraacetic acid (EDTA)] in the presence of a protease inhibitor cocktail (Roche). Cells were frozen and thawed 5×, sonicated on ice (4× for 10 seconds) and incubated for 10 min on ice, followed by centrifugation at 14,000 rpm for 1 h at 4°C. The supernatant was incubated with monoclonal anti-Ty antibodies for 1 h at 4°C on a wheel. Protein A-Sepharose CL-4B (GE Healthcare Life Sciences) was then added and the incubation continued for 1 h at 4°C. Immune complexes fixed on beads were washed 3× in Co-IP buffer. Finally, beads were resuspended into SDS–PAGE loading buffer supplemented with 100 μM DTT.

Metabolic labeling

HFF cells were heavily infected with freshly released parasites and washed several hours later. After 30 h, cells were incubated in methionine/cysteine-free DMEM (Sigma) for 1 h before incubation in DMEM containing 50 μCi [35S]-labeled methionine/cysteine (Hartmann Analytic GmbH) per ml for 18 h at 37°C. Co-IPs were then performed as described in the previous section.

Mass spectrometry

Samples obtained after co-IP assays were separated on a 12% SDS–PAGE gel and stained with Coomassie blue. Bands of interest were cut out from the gel and sent to the Proteomics Core Facility (Faculty of Medicine, Geneva, Switzerland) for analysis. Bands were destained with washing with 50% ceric ammonium nitrate (CAN) in 50 μM ammonium bicarbonate for 30 min. Gel pieces were then dried and subjected to protein digestion by trypsin and peptide extraction. MS and tandem mass spectrometry (MS/MS) were performed on a 4800 Proteomics Analyzer MALDI-TOF/TOF mass spectrometer (Applied Biosystems) according to the tuning procedures suggested by the manufacturer. Peak lists were generated with the Launch peak to MASCOT tools with the following settings: for the MS data, mass range 850–4000, peak density of maximum 20 peaks per 100 Da, minimal S/N ratio of 15, minimal area of 250, maximum peak of 50; for the MS/MS data, mass range 80–2000, peak density of maximum 50 peaks per 200 Da, minimal S/N ratio of 5, minimal area of 20 and maximum number of peak set at 200. Such acquired MS and MS/MS data were compared to the database using MASCOT search engine (http://www.matrixscience). In MASCOT, the combined PMF and MS/MS search was performed on Eukaryota entries present in Uni-ProtSPTR database (UniProtKB/Swiss-Prot Release 54.2 and UniProtKB/TEmBL Release 37.2, 1691 110 sequences). Search settings allowed one missed cleavage with the trypsin enzyme selected, one fixed modification (carboxymethylated cysteine) and a variable modification (oxidation of methionine).

Molecular modeling of TgMLC2–TgMyoD interaction and docking

PfMyoA–MTIP and PkMyoA–MTIP complex structures (2QAC and 2AUC) were used as templates to model the structures of TgMyoA, TgMLC1, TgMyoD and TgMLC2 (29). Only the 14 most C-terminal residues were used for modeling of TgMyoA (residues 806 to 819) and TgMyoD (residues 803 to 816). Residues 69 to 213 of TgMLC1 and residues 228 to 364 of TgMLC2 were used for homology models. All models were built using MODELLER (48). Flexible molecular docking was performed for TgMLC1/MyoA and TgMLC2/MyoD complexes using GOLD (47). Particle swarm optimization (PSO) technique was employed in combination with genetic algorithm to find the optimal protein–peptide complex. Similar results were obtained independently using RosettaDock (48).

Acknowledgments

We are grateful to N. Klages-Jemelin for the purification of NtMLC2 protein used to produce polyclonal antibodies. We thank Dr G. Van der Goot for providing the purified Aeromonas hydrophila aerolysin and Dr V. Currathers for sharing the Δku80Δhxgpmt strain. The vector pARL-GFP plasmid and pEThb vector were kindly provided by Dr B. Crabb and Dr A. Houdusse, respectively. We acknowledge the publicly available genome databases (ToxoDB and Wellcome Trust Sanger Institute) and especially Fiona Tomley and Arnab Pain. This work was supported by the Swiss National Foundation.
to D. S.-F. (FN3100A0-116722) and the Seventh Framework Programme (FP7/2007-2013) under grant agreement number 242095. D. S.-F. is an International Research Scholar of the Howard Hughes Medical Institute.

Supporting Information

Additional Supporting Information may be found in the online version of this article.

Figure S1: Apicomplexans possess a large repertoire of CaM-related proteins. Phylogenetic maximum likelihood tree of apicomplexan myosin light chain, calmodulin, centrin and related EF-hand-containing sequences. The tree topology shown is the best (ln likelihood = −18 472.93) of 10 trees generated with PHYLIP’s PROML using randomized sequence input orders. Bootstrap support values above 50% (of 100 replicates) from maximum likelihood (ML; PROML) and neighbor-joining (NJ; PROTDIST and NEWNEIGH) analyses are indicated above and below branches, respectively. Database accession numbers are indicated in the figure behind the species names, and the full species names (in alphabetical order) are as follows: B. bovis, Babesia bovis; C. hominis, Cryptosporidium hominis; C. muris, Cryptosporidium muris; C. parvum, Cryptosporidium parvum; E. tenella, Eimeria tenella; N. caninum, Neospora caninum; P. berghei, Plasmodium berghei; P. falciparum, Plasmodium falciparum; P. vivax, Plasmodium vivax; P. yoelii, Plasmodium yoelii; T. annulata, Theileria annulata; T. gondii, Toxoplasma gondii; T. parva, Theileria parva.

Figure S2: The localization of MLCs in intracellular parasites is not dependent on the position of the epitope tag. A) Schematic representation of the constructs used in this study, under the control of the tubulin promoter. Scale bar: 2 μm. B) Extracellular parasites stably expressing MLC1Ty or MLC2Ty were treated with 100 μM tachypleginA or an equivalent amount of dimethyl sulfoxide (DMSO) as a treatment control. MLC1 and MLC2 were revealed by Western blot using anti-Ty antibodies, whereas anti-GAP45 stained the periphery of the parasites. Scale bar: 2 μm.

Figure S3: TgMLC2–TgMyoD is a two-component complex, not modified upon tachypleginA treatment. A) Palmitoylation predictions for TgMLC2 and TgMyoD performed with CSS-PALM 2.0 at http://www.csspalm.biocuckoo.org/prediction.php. B) Extracellular parasites stably expressing MLC1Ty or MLC2Ty were treated with 100 μM tachypleginA and no EST or peptide; incomplete sequence performed with EMBOSS Pairwise Alignment (http://www.ebi.ac.uk/Tools/emboss/align). Expression results (ESTs and proteomic data) were obtained from ToxoDB, PlasmoDB and CryptoDB for all Apicomplexan species. Theileria species, PlasmoDB for Plasmodium species and CryptoDB for Cryptosporidium species.

Table S1: Separate excel file. The accession numbers are from ToxoDB for T. gondii, ToxoDB and Wellcome Trust Sanger Institute for N. caninum, Wellcome Trust Sanger Institute for E. tenella, B. bovis and Theileria species, PlasmoDB for Plasmodium species and CryptoDB for Cryptosporidium species.

Table S2: Comparison of MLCs sequences in Apicomplexan. The percent-identity and similarity is deduced from the comparison with T. gondii MLC1 corresponding sequence performed with EMBOSs Pairwise Alignment Algorithms (http://www.ebi.ac.uk/Tools/emboss/align). Expression results (ESTs and proteomic data) were obtained from ToxoDB, PlasmoDB and Wellcome Trust Sanger Institute databases. *, incomplete sequence; −, no EST or peptide; +, peptide identified.

Table S3: Identification of TgMyoD by mass spectrometry.

Table S4: Oligonucleotide primers used in this study for annotation and cloning. The restriction sites are underlined and non-coding sequences are shown in lowercase.

Appendix S1: Alignment of all the apicomplexan EF-hand-containing proteins used for the phylogenetic analysis. Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.

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References

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