Plasmodium falciparum possesses two GRASP proteins that are differentially targeted to the Golgi complex via a higher- and lower-eukaryote-like mechanism

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Summary

***Plasmodium falciparum*, the causative agent of malaria, relies on a complex protein-secretion system for protein targeting into numerous subcellular destinations. Recently, a homologue of the Golgi re-assembly stacking protein (GRASP) was identified and used to characterise the Golgi organisation in this parasite. Here, we report on the presence of a splice variant that leads to the expression of a GRASP isoform. Although the first GRASP protein (GRASP1) relies on a well-conserved myristoylation motif, the variant (GRASP2) displays a different N-terminus, similar to GRASPs found in fungi. Phylogenetic analyses between GRASP proteins of numerous taxa point to an independent evolution of the unusual N-terminus that could reflect unique requirements for Golgi-dependent protein sorting and organelle biogenesis in *P. falciparum*. Golgi association of GRASP2 depends on the hydrophobic N-terminus that resembles a signal anchor, leading to a unique mode of Golgi targeting and membrane attachment.**

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Introduction

Malaria is an infectious disease caused by protozoan parasites of the genus Plasmodium. Up to 500 million people are infected worldwide and more than one million people die from it each year, mostly children under the age of five years in sub-Saharan Africa (Butler, 2002). After an initial multiplication step in liver cells, the parasite invades and multiplies within red blood cells. To survive, the parasite extensively modifies its host cell by exporting parasite proteins to the host cell cytoplasm and to its cell surface. This requires efficient protein trafficking, not only within but also beyond the parasite boundaries in an environment that altogether lacks a secretion machinery.

The Golgi complex forms the centre of the secretory pathway and has a pivotal role in protein modification, processing and sorting (Short et al., 2005; Wang et al., 2005). Its unique feature is a stack of flattened cisternal membranes with three functionally distinct regions: the cis-, medial and trans-Golgi (Rothman and Orci, 1992; Wang et al., 2005). Golgi re-assembly stacking proteins (GRASPs; also known as GORASP1 and GORASP2) are peripheral membrane proteins that are present in all eukaryotic organisms except plants (Barr et al., 1998; Barr et al., 1997; Shorter and Warren, 2002; Short et al., 2001; Ward et al., 2001; Lane et al., 2002; Wang et al., 2003). A highly conserved myristoylation motif at the extreme N-terminus mediates Golgi-membrane association, and subsequent trans-oligomerisation of the protein leads to the characteristically stacked structure of Golgi cisternae (Barr et al., 2001; Short et al., 2005). Vertebrates have two *grasp* genes yielding two proteins that are named according to their molecular mass GRASP55 and GRASP65. They appear to define different membrane compartments within the Golgi stack (Barr et al., 1997; Shorter et al., 1999) and seem to be structurally important for the overall Golgi architecture (Sutterlin et al., 2005).

Non-myristoylated GRASP proteins have only recently been described for fungi (Behnia et al., 2007). It has been shown that, instead of N-myristoylation, N-terminal acetylation together with an amphipathic helix mediates membrane attachment in these species (Behnia et al., 2007). The malaria parasite *P. falciparum* possesses a GRASP homologue that uses N-terminal myristoylation for membrane attachment (Struck et al., 2005). This protein defines the *P. falciparum* Golgi complex as a distinct compartment in close proximity to the nucleus. Here, we report on a second GRASP protein derived from the same genetic locus by differential splicing. This isoform, hereafter referred to as GRASP2, lacks myristoylation as a membrane-attachment moiety but is instead characterised by a unique and fungi-like hydrophobic N-terminus that is necessary for its association with the Golgi membrane.

Results and Discussion

Two *grasp* mRNA populations that code for a myristoylated and non-myristoylated GRASP protein

The *P. falciparum grasp* gene was recently identified as a single-copy gene displaying a two-exon structure (Struck et al., 2005). Northern blot analysis on *P. falciparum* RNA using a probe specific to a 591-bp region within the *grasp* gene revealed two distinct transcripts of approximately 3.8 kb and 4 kb, suggesting alternative transcription initiation and variant splicing (Fig. 1A). This was further analysed by reverse transcribed RNA and subsequent PCR analysis. Using a 5′ *grasp*-specific oligonucleotide that binds to exon 1 and a *grasp*-specific antisense oligonucleotide, two distinct fragments with a size difference of ~150 bp were amplified (Fig. 1B). Appropriate controls were included to exclude contamination of the RNA preparation with genomic DNA (gDNA) (see Fig. S1 in supplementary material). Sequencing of PCR products revealed one spliced cDNA population, representing the *grasp* gene that has been previously described [grasp1, chr10.phat_187 (Struck et al., 2005)] and an additional non-spliced version encoding a GRASP variant with an alternative N-terminus (*grasp2*, Fig. 1D and data not shown). Although under standard SDS-PAGE conditions GRASP proteins appear as a single band, maximum separation on 7% SDS-PAGE and subsequent western blotting resulted in the detection of two GRASP proteins with a slightly different molecular mass (Fig. 1C; calculated molecular mass of GRASP1 is 66.8 kDa versus 68.4 kDa of GRASP2). Primary sequence analysis of the *grasp* gene revealed an in-frame alternative start ATG within the 148-bp intron. In accordance with our findings, the *grasp* intron has an unusual GC distribution: the GC contents of the non-coding 5′ intron end (9.7%) lies close to the average for introns (13.5%) (Gardner et al., 2002), whereas the putative protein-coding region within the intron lies well above that average (21% GC) and resembles the average GC-content of an exon (23.7%) (Gardner et al., 2002). Both GRASP proteins are identical except for their N-termini. Alternative translation initiation and alternative splicing are well known mechanisms to express different proteins from a single gene (Blencowe, 2006; Lu and Cidlowski, 2004). In *Plasmodium* spp. alternative splicing has been shown to occur for genes such as, for example, the cyclin-dependent kinase PK6 (Bracchi-Ricard et al., 2000), stromal-processing peptidase (van Dooren et al., 2002), adenylyl cyclase α (Muhia et al., 2003) and the invasion-related adhesive protein MAEBL (Singh et al., 2004). Importantly, GRASP2 does not possess a penultimate glycine, a prerequisite for N-terminal myristoylation (Boutin, 1997) but a hydrophobic stretch that is predicted to be a signal anchor by SignalP 3.0 (Bendtsen et al., 2004). Although the *grasp* intron-exon structure is well conserved among *Plasmodium* spp. only *Plasmodium vivax* displays a similar two-ORF-scenario (see Fig. S2 in supplementary material).

GRASP2 is characterised by a fungi-like N-terminal domain

N-terminal myristoylation ensures membrane attachment of GRASPs throughout the evolutionary tree of life (see Fig. S3 in supplementary material). They shuttle between a cytosolic pool and membrane association (Ward et al., 2001), although the precise mechanism of how these proteins are targeted to the Golgi complex remains unclear. Nevertheless, myristoylation is regarded as a prerequisite for association to the Golgi. Mutation of the myristoylation motif resulted in a cytosolic variant of *P. falciparum* GRASP1 (Struck et al., 2005). By contrast, *P. falciparum* GRASP2 displays no myristoylation motif but contains an N-terminal stretch

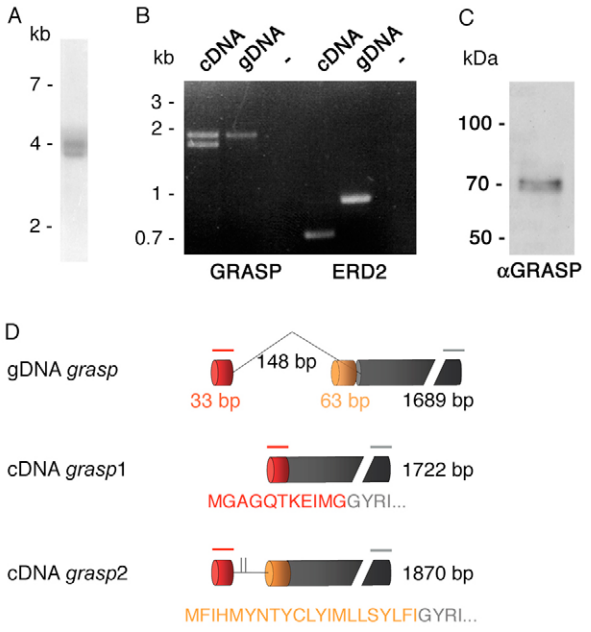

Fig. 1. Identification of two GRASP populations in *P. falciparum*. (A) Northern blot analysis using total RNA of wild-type parasites. A P32 labelled *grasp*-specific probe detects two transcripts of approximately 3.8 kb and 4 kb. (B) RT-PCR analysis. Two PCR products with a size difference of approximately 150 bp are amplified on cDNA (lane 1) using *grasp*-specific oligonucleotides. Genomic DNA (gDNA) was used as a positive control (lane 2). To exclude non-specific amplification reactions without template were run as negative controls (lanes 3 and 6). To exclude contaminations of the cDNA preparation with gDNA, *erd2*-specific oligonucleotides were used (lane 4-6). Consistent with the two-intron structure of the *erd2* gene (PF13_0280), a size difference is visible between cDNA (660 bp, lane 5) and gDNA (960 bp, lane 6). (C) Detection of two GRASP proteins in parasite extract. Maximum separation of parasite proteins and subsequent western blotting with GRASP-specific antibodies reveal two translation products of ~70 kDa. (D) Schematic representation of the genomic *grasp* gene and transcript heterogeneity. (Top) Exon 1 (red) encompasses 33 bp and is separated by a 148 bp intron from exon 2 (grey, 1689 bp). The intron possesses a putative start ATG and 63 bp ORF (yellow) in-frame with exon 2. (Middle and bottom) RT-PCR products were cloned and sequenced. Two different cDNA populations were identified and named cDNA grasp1 and cDNA grasp2, representing a spliced and unspliced version of the *grasp* gene. The deduced N-terminal amino acid sequence of GRASP2 is displayed in one-letter code in yellow and grey. Vertical line represents stop codons within the intron. Relative positions of oligonucleotides used in RT-PCR are presented as red and grey bars.

of hydrophobic amino acids that resembles a signal anchor sequence. Further, the N-terminus shows some similarities with those of fungi, where N-terminal acetylation of a phenylalanine residue is implicated in recruitment of GRASP homologues to the Golgi (see Fig. S3 in supplementary material) (Behnia et al., 2007). The unusual fungus-like *P. falciparum* GRASP2 N-terminus prompted us to analyse the phylogenetic relationship and N-terminal membrane-attachment signals of GRASP proteins in various taxa (Fig. 2). Primary sequence analysis and similarity searches of (in some cases putative) GRASP proteins confirmed the previous finding by Short and co-workers (Short et al., 2005) that GRASP is absent in plants. Our phylogenetic study recovered several major eukaryotic branches, i.e. the alveolata, metazoa and fungi, as well as a small number of trypanosomatid parasites representing the discicristata and some heterokont organisms (Baldauf, 2003). The microsporidian *Encephalitozoon cuniculi* is the only seriously misplaced taxon in the tree, but strong sequence divergence that

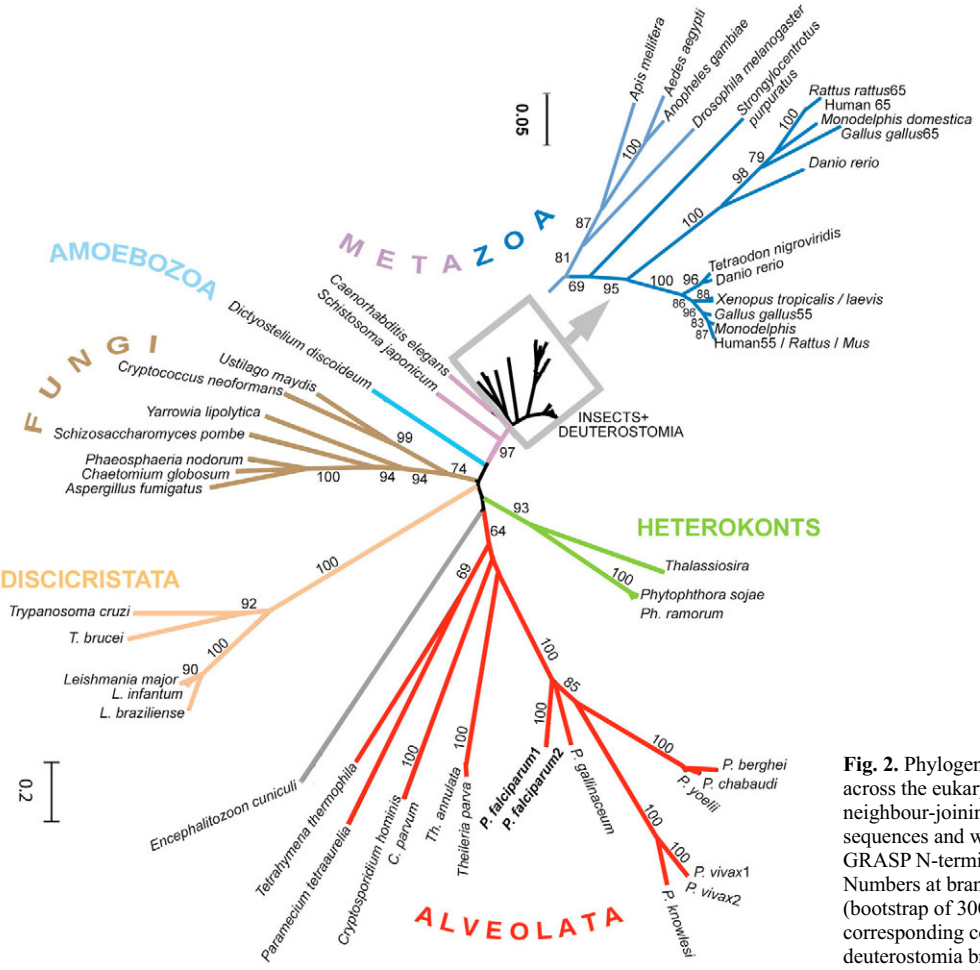

Fig. 2. Phylogenetic analysis of GRASP proteins across the eukaryotic tree of life. This unrooted neighbour-joining tree is based on 53 protein sequences and was estimated from an alignment of GRASP N-terminal domains (~370 amino acids). Numbers at branches indicate statistical support (bootstrap of 300 replicates) of >50% in the corresponding consensus tree. The insects and deuterostomia branch is shown at a magnified scale.

leads to erroneous positioning in phylogenetic trees is a well-established and frequently encountered problem with these organisms that are most closely related to the Fungi (Keeling, 2003). Within the metazoa GRASP55 and GRASP65 form two well-supported separate clades suggesting the descent from a gene duplication event in a vertebrate ancestor. The tree does not provide any significant evidence for a closer relationship of the Plasmodium GRASP proteins and their fungal homologues, despite their similar N-termini. This suggests an independent evolution of the fungi-like N-terminus in Plasmodium. Interestingly, the diplomonad *Giardia lamblia* appears to lack a *grasp* gene homologue, whereas the supposedly 'Golgi-less' *Cryptosporidium* (Tetley et al., 1998) does possess one. The presence of an N-terminal GRASP myristoylation motif appears to be conserved across all taxa except for some fungi [e.g. *S. cervisiae*, *A. fumigatus* and *Y. lipolytica* (Behnia et al., 2007)], the echinoderm *S. purpuratus* and the GRASP2 variants of *P. falciparum* and *P. vivax* (see Fig. S3 in supplementary material).

GRASP2 localises to the Golgi complex

To evaluate GRASP2 protein expression and subcellular localisation in the absence of a myristoylation motif, we generated an expression vector encoding the full-length *grasp* gene as a GFP-fusion protein and included a mutation in the 5′ splice site (GT to CA, Fig. 3A, pARL-GRASP1*/2-GFP). The latter should prevent GRASP1 expression because stop codons are present in the then unspliced intron, as well as leading to the sole expression of a GFP-tagged GRASP2 protein in the case of a functional translation initiation. Only a single transcript was produced from this plasmid as shown by reverse transcriptase (RT)-PCR from parasites harbouring pARL-GRASP1*/2-GFP (Fig. 3B). This is in contrast to an expression vector that encodes the full-length *grasp* gene without a mutation in the 5′ splice site (see Fig. S4A in supplementary material), for which both transcripts were detectable by RT-PCR (see Fig. S4B in supplementary material). Whereas anti-GRASP antibodies detect the endogenous protein of ~70 kDa in both wild-type and pARL-GRASP1*/2-GFP-transfected parasites, an additional protein of ~100 kDa that represents the GRASP2-GFP was recognised exclusively in transgenic parasites (Fig. 3C). Thus, despite a functionally inactivated exon 1, the GRASP2-GFP fusion protein is still expressed, confirming recognition of the translation initiation site in the unspliced transcript and the expression of the GRASP isoform.

To evaluate the subcellular localisation of GRASP2 we investigated pARL-GRASP1*/2-GFP transfected parasites either by fluorescence microscopy of live cells or indirect immunofluorescence in combination with different antibodies on fixed parasites. GRASP2-GFP was found in tightly defined compartments within the parasite juxtaposed to the nucleus with minimal cytoplasmic background fluorescence (Fig. 3E). This fluorescence pattern resembled the distribution of GRASP1 as reported previously (Struck et al., 2005). One single GRASP2-GFP compartment in close proximity to the nucleus was observed

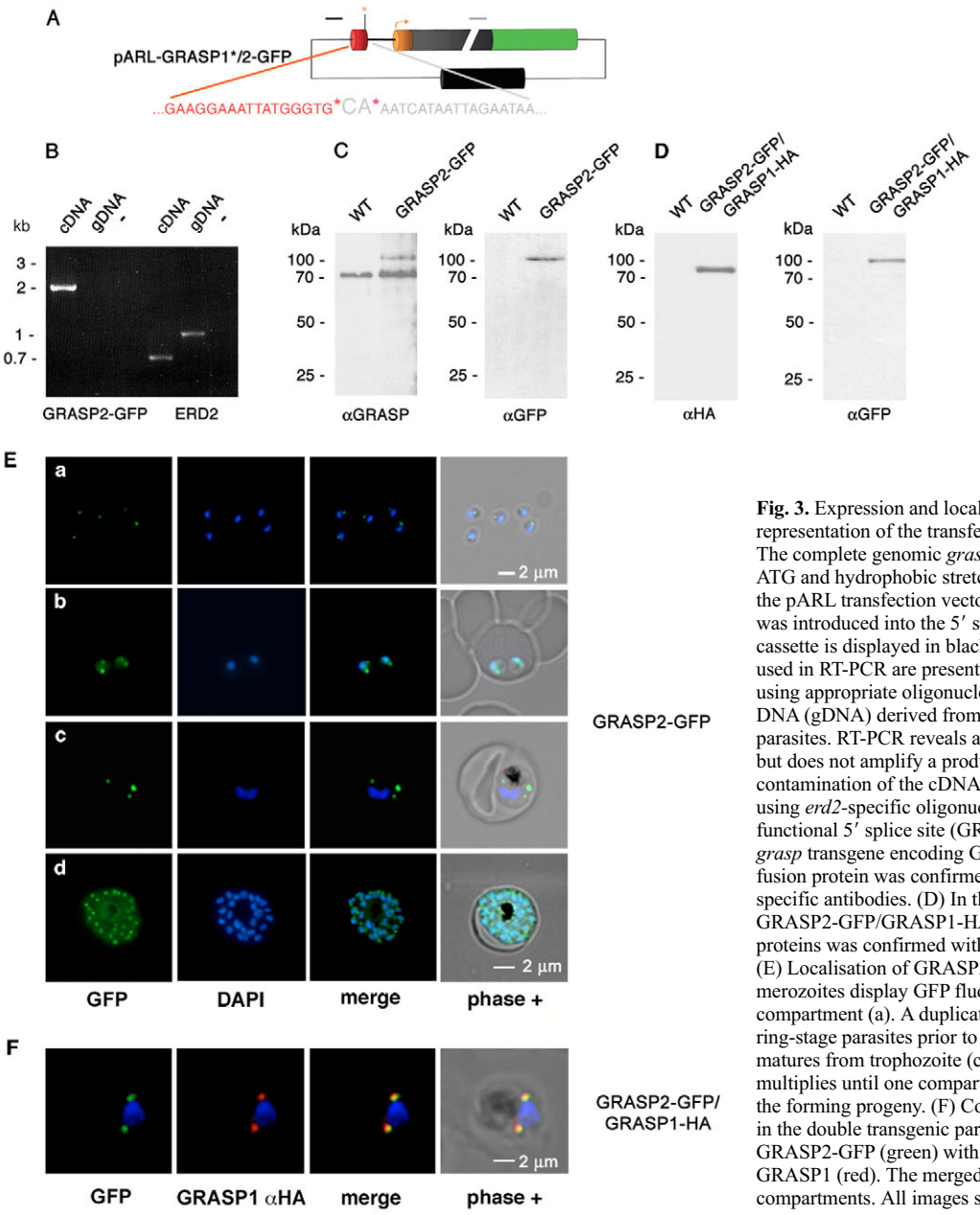

Fig. 3. Expression and localisation of GRASP2 (A) Schematic representation of the transfection vector pARL-GRASP1*/2-GFP. The complete genomic *grasp* sequence (exon 1: red; alternative start ATG and hydrophobic stretch: orange; exon 2: grey) was cloned into the pARL transfection vector in frame with GFP (green). A mutation was introduced into the 5′ splice site (*). The human *Dhfr* selection cassette is displayed in black. Relative positions of oligonucleotides used in RT-PCR are presented as bars. (B) Transcriptional analysis using appropriate oligonucleotides and either cDNA or genomic DNA (gDNA) derived from pARL-GRASP1*/2-GFP-expressing parasites. RT-PCR reveals a single PCR product using cDNA (lane 1) but does not amplify a product on gDNA (lane 2). The absence of any contamination of the cDNA preparation with gDNA was confirmed using *erd2*-specific oligonucleotides (lane 4-5). (C) Disruption of the functional 5′ splice site (GRASP1*/2-GFP) leads to translation of a *grasp* transgene encoding GRASP2-GFP. Expression of the GFP fusion protein was confirmed with either anti-GRASP or anti-GFP specific antibodies. (D) In the double transgenic parasite line GRASP2-GFP/GRASP1-HA simultaneous expression of the fusion proteins was confirmed with either GFP- or HA-specific antibodies. (E) Localisation of GRASP2-GFP in unfixed parasites. Free merozoites display GFP fluorescence in one tightly defined compartment (a). A duplication of this compartment takes place in ring-stage parasites prior to nuclear division (b). As the parasite matures from trophozoite (c) to schizont (d) the organelle further multiplies until one compartment can be equally distributed among the forming progeny. (F) Colocalisation of the two GRASP proteins in the double transgenic parasite line. Immunofluorescence assay of GRASP2-GFP (green) with HA-specific antibodies representing GRASP1 (red). The merged image shows colocalisation of the two compartments. All images show the nucleus in blue (DAPI).

in free merozoites (Fig. 3Ea). Duplication of the GRASP2-defined compartment occured prior to nuclear division (Fig. 3Eb) resulting in a multiplicity of Golgi compartments in schizonts (Fig. 3Ec,d). Colocalisation studies using antibodies specific for the endoplasmic reticulum (ER) marker BiP (Kumar et al., 1991) and the cis-Golgi marker ERD2 (Elmendorf and Haldar, 1993; Struck et al., 2005) showed that the distribution of GRASP2-GFP resembles that of its myristoylated counterpart GRASP1 (see Fig. S5A,B in supplementary material). Additionally, we generated a parasite line that expresses GRASP2 with a C-terminal TY1-tag to exclude any influence of the GFP reporter on the subcellular localisation of the protein (see Fig. S6 in supplementary material). In agreement with our previous results GRASP2-TY1 localised to a compartment in close proximity to the nucleus and colocalised with ERD2 (see Fig. S6 in supplementary material and data not shown).

To allow a direct colocalisation of the two GRASP proteins we generated a double transgenic parasite line expressing GFP-tagged GRASP2 and haemagglutinin (HA)-tagged GRASP1. Expression of the fusion proteins was confirmed in western blot analyses using GFP- and HA-specific antibodies (Fig. 3D). Immunofluorescence assays showed colocalisation of GRASP 1 and GRASP2, thus both variants reside in the Golgi (Fig. 3F).

The N-terminal domain of GRASP2 is responsible for its association with the Golgi complex

To analyse the sequence requirements for the recruitment of GRASP2 to the Golgi compartment two N-terminal deletion mutants were generated (GRASP2ΔSA-GFP and GRASP2ΔSA2-GFP), which lack the signal anchor (SA) sequence. Expression of the transgenes was confirmed by western bloting using GFP-specific antibodies (Fig. 4A). Deletion of the N-terminal hydrophobic 21

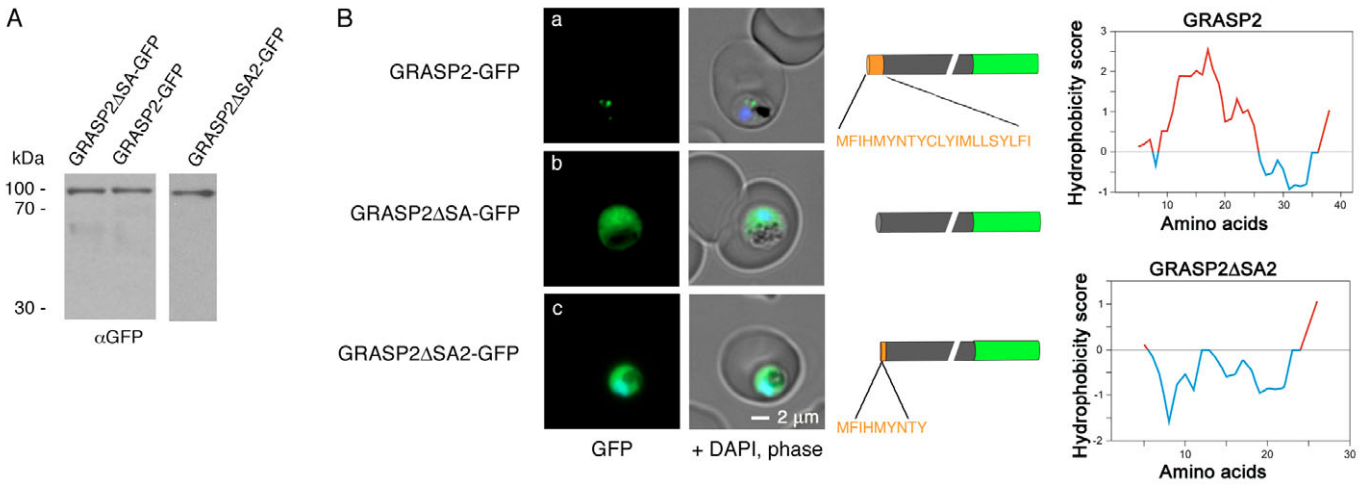

Fig. 4. Distribution of GRASP2 depends on its hydrophobic N-terminus. (A) Expression of the N-terminal deletion mutants GRASP2ΔSA-GFP and GRASP2ΔSA2-GFP was confirmed using GFP-specific antibodies. A single band of ~100 kDa resembling the GFP-fusion protein is recognised in all transgenic parasite lines. (B) Fluorescence microscopy on live parasites that express either GRASP2-GFP or the N-terminal deletion mutants GRASP2ΔSA-GFP and GRASP2ΔSA2. Whereas GRASP2-GFP is restricted to tightly defined compartments (a), mutation of the N-terminus either by complete deletion of the hydrophobic stretch (b) or partial removal of its proximal part leaving the extreme N-terminus intact (c) abolishes Golgi targeting and results in a cytoplasmic distribution of the fusion protein. All images show the nucleus in blue (DAPI).

amino acids that comprise the putative signal anchor (GRASP2ΔSA-GFP) resulted in the loss of Golgi localisation and expression of a cytosolic variant (Fig. 4Bb). The same localisation phenotype was generated by deletion of 12 amino acids in the second half of the putative signal-anchor sequence (Fig. 4Bc). This mutation preserves the extreme N-terminus, including a conserved phenylalanine that is implicated in acetylation of fungi GRASP proteins (Behnia et al., 2007) (see Fig. S3 in supplementary material) but changes the hydrophobicity of the region. This indicates that the N-terminal hydrophobic domain is essential for GRASP2 recruitment to the Golgi and represents a new mechanism for membrane association of GRASP proteins. It represents a functional equivalent to the myristoylation motif of GRASP1 but precludes shuttling from a cytoplasmic pool to the Golgi membrane. This is reflected in the solubility properties of the two proteins: compared with GRASP2-GFP, myristoylated GRASP1-GFP is less tightly associated with membranes (see Fig. S7 in supplementary material), which points towards a more solid membrane association of GRASP2 (conferred by its hydrophobic N-terminus) than N-terminal myristoylation, which is known to rely on additional sequence features for strong membrane binding (Peitzsch and McLaughlin, 1993). However, it should be noted that additional means of Golgi-specific GRASP2 recruitment, potentially involving acetylation of the conserved phenylalanine residue, cannot be excluded.

Implications of a second GRASP protein for Golgi function

GRASP proteins can be found in most major systematic groups, such as alveolata, discicristata, heterokonts, amoebozoa, fungi and metazoa (Richards and Cavalier-Smith, 2005), and appear to be absent only in plants and diplomonads (Fig. 2). This suggests that, (1) in eukaryotic evolution an early eukaryotic ancestor possessed a Golgi complex (Dacks and Doolittle, 2001; Helenius and Aebi, 2001; Shorter and Warren, 2002) that was already organised by GRASP proteins and, (2) during plant and diplomonad evolution this protein was either lost or greatly modified. In the vertebrate lineage the *grasp* gene was duplicated, yielding the two protein variants (GRASP55 and GRASP65) whereas in all other organisms and, notably, in the various protozoan lineages GRASP proteins appear to be encoded by a single gene (Fig. S2 and Fig. S3 in supplementary material). The intron-exon structure is well-conserved within the Plasmodium genus (see Fig. S2 in supplementary material) although only *P. vivax* and *P. falciparum* possess an alternative ATG-start site within the intron. Given the evolutionary distance of Plasmodium GRASP to their fungal counterparts we conclude that the Plasmodium variants without a myristoylation motif (*P. falciparum* GRASP2 and putative *P. vivax* GRASP2) evolved independently from the fungal proteins in convergent events. It is tempting to speculate that the occurrence of a non-myristoylated GRASP variant in *P. falciparum* (and potentially *P. vivax*) reflects an evolutionary adaptation of this human pathogen to live up to unique requirements in protein modification and sorting.

Vertebrates possess two *grasp* genes; they encode two proteins that are homologous but localise to different parts of the Golgi stack and interact with different proteins. Given the existence of two GRASP proteins in the malaria parasite *P. falciparum*, it is interesting to speculate as to why a parasite with a seemingly simple and 'unstacked' Golgi architecture (Bannister et al., 2003; Elmendorf and Haldar, 1993; Van Wye et al., 1996) evolved to express a second GRASP protein. The appearance of GRASP variants could argue for a more complex Golgi organisation than suspected so far, which might be revealed by higher-resolution-imaging techniques. Alternatively, the GRASP variants might be implicated in organelle replication. In this scenario membrane anchored GRASP2 might form an initial Golgi matrix, thereby enabling recruitment of a second recyclable matrix component (GRASP1) to ensure rapid template-assisted Golgi biogenesis during the extremely efficient parasite multiplication steps in liver and red blood cells.

Materials and Methods

Cell culture and transfection of *P. falciparum*

P. falciparum asexual stages (3D7) were cultured and transfected as described previously (Trager und Jansen, 1976; Wu et al., 1995; Fidock and Wellems, 1997; Struck et al., 2005). Positive selection for transfectants was achieved using 10 nM

WR99210 (Fidock and Wellems, 1997). The double transgenic cell line GRASP2-GFP/GRASP1-HA was generated by transfecting the GRASP2-GFP-expressing cell line with the vector pBcamR-GRASP1-HA and selecting parasites with 30 nM WR99210 and 2 μg/ml BlastocidinS (Roche).

Phylogenetic analysis of GRASP proteins

The conserved, N-terminal GRASP domain of a total of 53 taxonomic units was used for phylogenetic analysis. Sequences were retrieved from Joint Genome Institute (http://genome.jgi-psf.org), PlasmoDB (www.plasmoDB.org) or GenBank (see Fig. S2 in supplementary material). To construct the phylogenetic tree, the ClustalX-generated alignment (a dataset of 370 amino acids) was entered into the phylogenetic analysis program PHYLIP (Phylogeny Inference Package) Version 3.66 for MacOSX, using the program components 'seqboot', 'protdist', 'neighbour' and 'consense' (http://evolution.genetics.washington.edu/phylip.html). Distance matrices were calculated employing the Jone-Taylor-Thornton matrix in 'protdist', and phylogenetic trees were inferred by the neighbour-joining method using 'neighbour'. For bootstrapping (300 replicates) the dataset was re-sampled with 'seqboot' and an unrooted 50% majority-rule consensus tree was generated with 'consense'. The consensus tree was subsequently redrawn using MEGA version 3.0 (Kumar et al., 2004).

Northern blot analysis, RT-PCR and nucleic acids

Total RNA was isolated from parasites using Trizol (Invitrogen). Approximately equal amounts were loaded onto a 1.1% Agarose Gel (Ambion) and transferred onto a Hybond XL Nylon Membrane (Amersham). Northern blots were hybridised with a 591 bp *grasp* fragment radiolabelled with ^{32}P [amplified with the primer pair: graspN5′-sense (-S) and graspN5′-antisense (-AS) (supplementary material Table S1)] in Ultrahyb hybridisation buffer (Ambion) at 42°C and washed twice in 2×SSC at 62°C. Fuji Medical X-Ray films were used for detection.

For RT-PCR and cloning of the alternative cDNAs of the *grasp* gene, total RNA was isolated from parasites using Trizol. RNA was DNase digested. Single-strand cDNA was synthesised from approximately 1 μg of total RNA with 'Superscript' and random hexamers (Invitrogen). PCR on wild-type cDNA was carried out using the *grasp*-specific primers grasp-S and grasp-AS (supplementary material Table S1). To exclude contamination of gDNA, five additional intron-containing genes (PF13_0280, PF13_0082, PF11_0164, PF14_0119, PFB0570w) were amplified using gene-specific primers (supplementary material Table S1). PCRs on transgenic cDNAs of GRASP1/2 (see Fig. S1 in supplementary material) and GRASP1*/2-GFP (Fig. 3A) parasite lines were performed using either a sense primer binding within the 5′ UTR of the transgene (chloroquine-resistance transporter 5′UTR) or grasp-S in combination with the grasp-AS (data not shown). PCR products were cloned directly into pCR-TOPO (Invitrogen) and sequenced.

To obtain a transfection vector with a splice-site mutation the *grasp* gene was amplified using the sense primer grasp1*/2-S and grasp-AS (supplementary material Table S1) in a PCR reaction with gDNA. The amplified *grasp* mutant (grasp1*/2) was cloned into the transfection vector pARL-GFP in order to express GRASP2-GFP. The grasp1*/2 PCR products were digested with *Kpn*I and *Avr*II, and subsequently cloned into pARL-GFP (Struck et al., 2005). Additionally, GRASP2 was tagged with a C-terminal TY1 epitope (Bastin et al., 1996). The TY1 tag was introduced in a PCR reaction using the primer combination grasp2-S and grasp-AS-TY1 (supplementary material Table S1) resulting in the expression vector pARL-GRASP2-TY1. To allow expression of GRASP1 with a C-terminal HA-tag in the GRASP2-GFP-expressing parasite line, GRASP2 was cloned into the transfection vector pCamR3xHA (Christian Flück and Till Voss, Swiss Tropical Institute, Basel, Switzerland) using the primer grasp1-pBcamR-S and grasp1-pBcamR-AS (supplementary material Table S1). This transfection vector allows positive selection of transfected parasites with blastocidin. The GRASP2 N-terminal deletion mutants were generated using the sense primer grasp2-ΔSA or grasp2-ΔSA2 in combination with grasp-AS (supplementary material Table S1). The PCR fragments were cloned into pARL-GFP and transfected into parasites.

To confirm expression of both *grasp* variants in the parasite a transfection vector (pARL-GRASP1/2, supplementary material Fig. S3) was generated using the Myc-tag coding sequence introduced into exon 1 (resulting in Myc-tagged GRASP1) and the HA-tag sequence introduced into the GRASP2-specific sequence (resulting in HA-tagged GRASP2) using gDNA, Vent Polymerase (New England Biolabs) and appropriate primers (supplementary material Table S1) in an overlapping PCR approach. The PCR fragment was digested with *Kpn*I and *Xho*I and cloned into pARL1a– (Crabb et al., 2004).

Antisera and immunoblots

Anti-peptide rabbit antisera were raised against BiP (PlasmoDB PFI0875) using the peptide SGDEDVDSDEL as previously described (Kumar et al., 1991). Other primary antibodies used in immunodetection were rabbit anti-*P. falciparum* GRASP (Struck et al., 2005), rabbit anti-*P. falciparum* ERD2 (kindly provided by the Malaria Research and Reference Center, NIH, MRA-72; accession number NP705420) (Bannister et al., 2003; Elmendorf and Haldar, 1993; Van Wye et al., 1996), monoclonal anti-GFP, rabbit anti-HA, monoclonal anti-Myc (all Roche) and monoclonal anti-TY1 (Diagenode). Immunoblots were performed and developed as previously described (Struck et al., 2005). To separate the two GRASP variants, parasite extracts were separated on 7% SDS-PAGE minigels. Secondary antibodies were sheep anti-rabbit IgG horseradish peroxidase (Sigma) and sheep anti-mouse IgG horseradish peroxidase (Roche).

Immunofluorescence and analysis of GFP-expressing parasites

Immunofluorescence assays were performed on fixed parasites as previously described (Tonkin et al., 2004). Primary antibody dilutions in 3% BSA were 1:1000 for rabbit anti-*P. falciparum* GRASP and 1:2000 for rabbit anti-PfBiP, 1:10,000 for mouse anti-TY1 and 1:500 for rabbit anti-PfERD2. Cells were incubated 1:2,000 with Alexa-Fluor-594 goat anti-rabbit IgG antibodies (Molecular Probes) and 1:1000 with DAPI (Roche). Images of GFP-expressing parasites and immunofluorescence assays were observed and captured using a Zeiss Axioskop 2plus microscope, a Hamamatsu Digital camera (Model C4742-95) and OpenLab software version 4.0.4 (Improvision Inc.).

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