Re-defining the Golgi complex in *Plasmodium falciparum* using the novel Golgi marker *Pf*GRASP

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Summary

***Plasmodium falciparum,* the causative agent of malaria, relies on a sophisticated protein secretion system for host cell invasion and transformation. Although the parasite displays a secretory pathway similar to those of all eukaryotic organisms, a classical Golgi apparatus has never been described. We identified and characterised the putative Golgi matrix protein *Pf*GRASP, a homologue of the Golgi re-assembly stacking protein (GRASP) family. We show that *Pf*GRASP is expressed as a 70 kDa protein throughout the asexual life cycle of the parasite. We generated *Pf*GRASP-GFP-expressing transgenic parasites and showed that this protein is localised to a single, juxtanuclear compartment in ring-stage parasites. The *Pf*GRASP compartment is distinct from the ER, restricted within the boundaries of the parasite and colocalises with the cis-Golgi marker ERD2. Correct subcellular localisation of this Golgi matrix protein depends on a cross-species conserved functional myristoylation motif and is insensitive to Brefeldin A. Taken together our results define the Golgi apparatus in *Plasmodium* and depict the morphological organisation of the organelle throughout the asexual life cycle of the parasite.**

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Introduction

The intracellular parasite *Plasmodium falciparum* is the causative agent of malaria and responsible for over two million deaths each year (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 the host cell by exporting parasitic proteins to the host cell cytoplasm and to its cell surface. It has recently been shown that protein transport from the parasite via the surrounding vacuole and into the host cell involves a pentameric motif conserved across the genus *Plasmodium* (Marti et al., 2004; Hiller et al., 2004). Secreted proteins are synthesised in the parasite cytoplasm and translocated into the ER. This is mediated through a classical hydrophobic leader sequence, although some can be considerably recessed (Papakrivos et al., 2005). In addition, a functional retrieval mechanism for soluble proteins such as *Pf*BiP via the KDEL receptor *Pf*ERD2 has been demonstrated in this parasite (Elmendorf and Haldar, 1993a; Van Dooren et al., 2005). However, morphological and functional evidence for a Golgi apparatus and post-Golgi transport pathways in *Plasmodium* is still rather sparse. The Golgi apparatus is the central hub of the eukaryotic secretory machinery and plays a pivotal role in protein modification, processing and sorting. In general, the Golgi is organised into three functionally distinct regions: the cis-Golgi network (entry face), the Golgi stack and the trans-Golgi network (exit face). The ordered structure of this organelle is believed to reflect the requirement for the processing machinery to be compartmentalised for a sequential series of modification and sorting events (Farquhar and Palade, 1998).

The Golgi of protists offers an interesting insight into variations of the structure and organisation of this organelle. For example, representatives of two early-diverged eukaryotic lineages exhibit entirely distinct Golgi morphology and function: the diplomonad parasite *Giardia intestinalis* appears to have no stable Golgi compartment and there is no evidence for abundant protein modifications (Marti et al., 2003), whereas *Trypanosoma brucei* (kinetoplastids), the causative agent of sleeping sickness in humans, shows a classical stacked Golgi and high abundance of N-glycosylation in surface proteins (He et al., 2004). Further, *Toxoplasma gondii*, an apicomplexan parasite related to *Plasmodium*, has a single stacked Golgi apparatus as part of a highly polarised secretory pathway (Hager et al., 1999; Joiner and Roos, 2002; Pelletier et al., 2002). By contrast, morphology, organisation, function and even localisation of the Golgi in *Plasmodium* are still controversially discussed (Lingelbach, 1993; Elmendorf and Haldar, 1993b; Benting et al., 1994; Mattei et al., 1999; Bannister et al., 2000). Immunofluorescence and electron microscopy have not as yet provided unambiguous results as to the nature of the Golgi (Aikawa, 1971; Elmendorf and Haldar, 1993a; Van Wye et al., 1996; Bannister et al., 1990; Bannister et al., 2000;

Bannister et al., 2003). For instance, it was shown that *Pf*ERD2, a Golgi marker protein that in mammalian systems is concentrated in the cis-Golgi, is localised to the perinuclear region of the parasite (Elmendorf and Haldar, 1993a; Van Wye et al., 1996; Noe et al., 2000). It was also shown that the distribution of the trans-Golgi marker *Pf*Rab6 (Novick and Brennwald, 1993) in the parasite is distinct from *Pf*ERD2 (De Castro et al., 1996; Van Wye et al., 1996). It was concluded that in early blood stages the parasite displays a functional but primitive, unstacked Golgi with distinct compartments (Van Wye et al., 1996). This data was partially supported by electron microscopy. A typically stacked Golgi apparatus has not been identified in the parasite, but a discoid cisterna close to the nucleus has been described that was provisionally specified as a minimal Golgi apparatus (Bannister et al., 2000; Bannister et al., 2003). Previous fixation methods for *Plasmodium* have resulted in very poor preservation for all membranous structures and organelles, so the characterisation of the Golgi in fixed *Plasmodium* parasites must be treated with caution. To overcome the problem of poor organellar fixation, we used live-cell microscopy to visualise the Golgi apparatus.

In the present study, we characterised the *P. falciparum* orthologue of the Golgi re-assembly stacking protein (GRASP). GRASP proteins are peripheral membrane proteins involved in stacking of Golgi cisternae (Barr et al., 1997; Barr et al., 1998; Shorter et al., 1999). They are conserved from yeast to mammals and are Golgi-defining matrix proteins. Here we show that *Pf*GRASP is expressed in blood stage parasites as a 70 kDa protein. It is localised to a compartment juxtaposed to the nucleus. Using green fluorescence protein (GFP)-tagged chimeric proteins we analysed morphology and development of the Golgi apparatus in vivo during the life cycle of the parasite. We also show that targeting of *Pf*GRASP depends on a functional N-terminal myristoylation motif. In addition we used indirect immunofluorescence microscopy and specific antibodies against other Golgi marker proteins to analyse the spatial organisation and compartmentalisation of the Golgi apparatus in *Plasmodium*.

Materials and Methods

Cell culture and transfection of *P. falciparum*

Plasmodium falciparum asexual stages (3D7) were cultured in human 0^+ erythrocytes according to standard procedures (Trager and Jensen, 1976). 3D7 parasites were transfected as described previously (Wu et al., 1996) with 100 μg purified plasmid DNA (Qiagen). Positive selection for transfectants was achieved using 15 nM WR99210, an antifolate that selects for the presence of the human *dhfr* gene (Fidock and Wellems, 1997).

Nucleic acids and constructs

*Pf*GRASP was identified using the online BLAST tool implemented in the *Plasmodium* database (www.plasmodb.org). The cDNAs encoding *Pf*GRASP and *Pf*ERD2 (PlasmoDB PF13_0280) were generated using total RNA of parasites in a reverse-transcription reaction (Invitrogen) with specific oligonucleotides. Subsequently, the genes were PCR amplified with Vent Polymerase (Stratagene) and sequenced to detect unwanted mutations. The following primers were used: *Pf*GRASP-S, 5′-GCGC**GGTACC**ATGGGAGCAGGACAAACG-3′ and *Pf*GRASP-AS, 5′-GCGC**CCTAGG**CAATATGTTCTTTCTTAC-3′; *Pf*ERD2-S, 5′-GCGC**GGTACC**ATGAATATATTTAGACTG-3′ and *Pf*ERD2-AS, 5′-GCGC**CCTAGG**TTTTACTTCACCATTAAATG-3′.

To generate transfection vectors expressing C-terminal GFP fusion proteins, PCR products were digested with *Kpn*I and *Avr*II (bold) and cloned into pARL1a$^-$ (Crabb et al., 2004). GFP was previously inserted into the *Xho*I site of pARL1a$^-$ with an additional 5′ *Avr*II site. To alter the putative myristoylation site in *Pf*GRASP we changed the amino acid glycine at position 2 to alanine by using the sense primer (*Pf*GRASP-G_2/A-GFP: 5′-GCGC**GGTACC**ATGG**C**AGCAGGACAAACG-3′) and fused the fragment in-frame with GFP as described above.

Antisera and immunoblotting

Mouse antisera were raised against synthetic polypeptides of *Pf*GRASP (N_{529}SSKMDNITKGTYIN_{543}) and *Pf*Rab6 (PlasmoDB, PF11_0461, N_{181}EANVVDIQLTNNSNKND_{197}). Other antibodies used in immunodetection were rabbit anti-*Pf*ERD2 (obtained through the Malaria Research and Reference Center, NIH, MRA-72; accession number NP705420) (Elmendorf and Haldar, 1993a), monoclonal anti-GFP (Roche) and anti-*Pf*BiP (Malaria Research and Reference Center, MR-19; AAA29501) (Kumar et al., 1991). For immunoblots, parasite proteins from a synchronised culture were separated on 10% SDS-PAGE gels and transferred to nitrocellulose membranes (Schleicher & Schuell). Anti-*Pf*GRASP was diluted 1:1500 in phosphate-buffered saline (PBS). The secondary antibody was sheep anti-rabbit IgG horseradish peroxidase (Sigma) and used at a 1:3000 dilution. The immunoblots were developed by chemiluminescence using ECL (Amersham).

Immunofluorescence and analysis of GFP-expressing parasites

Green fluorescence of GFP-expressing transfectant cell lines was observed and captured in live cells through the erythrocytic life cycle every 8 hours using a Leica Axioskop 2 and OpenLab software (Improvision) or a Leica confocal microscope (TCS SP2) and Leica confocal software (LCS). Immunofluorescence assays were performed on fixed parasites as previously described (Tonkin et al., 2004). The primary antibody dilutions used in 3% BSA: rabbit anti-*Pf*ERD2 (1:500), rabbit anti-*Pf*BiP (1:1000), mouse anti-*Pf*GRASP (1:1000) and mouse anti-*Pf*Rab6 (1:1000). The cells were incubated with Cy3 anti-mouse IgG antibodies (Molecular Probes), Alexa-594 goat anti-rabbit IgG antibodies (Molecular Probes) and DAPI (1:1000, Roche). Dual-colour fluorescence images were captured using a Leica Axioskop 2 microscope or a Leica confocal microscope.

Brefeldin A treatment

Synchronised parasites were incubated with 5 μM Brefeldin A from a 10 mM ethanol stock solution. Control parasites were incubated with ethanol to exclude any morphological alterations due to the presence of ethanol. Brefeldin A was removed after 24 hours and the parasites were cultured for another 24 hours to ensure viability after treatment.

Real-time RT-PCR

Synchronised parasites were harvested at 8 hour intervals and total RNA was isolated using Trizol (Invitrogen). Total RNA (1 μg) was reverse transcribed and quantified in a LightCycler (Roche) using Quantitect SYBR Green RT-PCR Kit (Qiagen) and gene-specific primers. Reaction conditions were 50°C for 20 minutes, 95°C for 15 seconds, 55°C for 20 seconds and 72°C for 20 seconds. A stage-specific control was performed using the early upregulated *etramp* gene (early-transcribed membrane protein; PlasmoDB PF10_0019) (Spielmann et al., 2003). Serial dilutions of 3D7 total RNA were used

as a standard reference control. The relative transcription ratios through the asexual life cycle of *grasp* and *etramp* compared with the reference gene *actin* (PlasmoDB PFL2215w) were calculated. Primers used were as follows: *actin*-S, 5′-TGCACCACCAGAGAGAAAAT-3′; *actin*-AS, 5′-ACTTGGTCCTGATTCATCGT-3′; *etramp*-S, 5′-ATGTTTTAGGAGGTAGTAGTGGATCAG-3′; *etramp*-AS, 5′-GCGAGAACAGAAGCTACGGAAG-3′; *grasp*-S, 5′-AAAGTGCAAATTCAACTTAATGATTCA-3′; *grasp*-AS, 5′-TCTTTAATTTGACAAGGTGGAATTTTATG-3′.

Results

Identification of the Golgi-stacking protein *Pf*GRASP and primary sequence analysis

A few intrinsic Golgi markers are conserved across the eukaryotic lineage, the most prominent ones being members of the GRASP family. We identified a single *Pfgrasp* homologue in the genome of the parasite (chr10.phat_190, www.plasmodb.org). The gene is located on chromosome 10, encompasses 1869 base pairs in length and encodes a 574 amino acid protein. Contrary to the annotation (one-exon structure; PlasmoDB PF10_0168), *Pf*GRASP is encoded by a two-exon gene, whereas the first exon encodes the first 11 amino acids. This short N-terminal extension displays a cross-species conserved putative myristoylation site (NMT MYR Predictor) (Bologna et al., 2004) that might facilitate membrane attachment (Fig. 1A,B). Cloning and sequencing of the gene from both genomic DNA and cDNA isolated from infected red blood cells confirmed the gene structure (data not shown). The *Plasmodium* protein displays an overall identity of 22% with human and rat (*Rattus norvegicus*) GRASP55 and 19% identity with the GRASP protein identified in the related apicomplexan parasite *Toxoplasma gondii* (Pelletier et al., 2002) (Fig. 1B). The homology is restricted to the N-terminal 220 amino acids. This domain is implicated in dimerisation and trans-oligomerisation in mammalian GRASP proteins (Barr et al., 1998; Wang et al., 2003; Wang et al., 2005). *Pf*GRASP has a M_r of 69×10^3 and is larger than all other GRASP proteins. This is due to an ~120 amino acid extension of the unconserved C-terminal domain of *Pf*GRASP (residues 219-573). Although these enlargements of proteins (either

A.

B.

```
RnGRASP55       MGSSQSVEI PG--- ----G GTEGY HVLRV QENSPG HRAGL EPFFD FIVSI SGSRL NKDND  53
HsGRASP55       MGSSQSVEI PG--- ----G GTEGY HVLRV QENSPG HRAGL EPFFD FIVSI NGSRL NKDND  53
TgGRASP         MGAASSLDQ RGTLA VGEKA RGGAY RVVKV QRGSPA EEAGL EVFFD FITRI DDLPL TSPNE  60
PfGRASP         MGAGQTKEI MG--- ----- ---GY RILRI SENSPC SNVGL EIFFD YIIQI DDLKL LDSSK  49
                **:..: : *            .*::: ::... **  . .*** ***:*  *..  * . ..

RnGRASP55       TLK DLLKA NVEK- ----PV KMLIY SSKTL ELREA SVTPS NLWGG QG--- ----L LGVSI R 101
HsGRASP55       TLK DLLKA NVEK- ----PV KMLIY SSKTL ELRET SVTPS NLWGG QG--- ----L LGVSI R 101
TgGRASP         ETL QAFFA KVNRS TRPEPV QLIVF NARMR GFRNV TLRPA VLSHA QRPAA SCVFS LGLSV S 120
PfGRASP         RTY DNFIE KIKLH EN-KEL TLDIY NCRYD KIKKV KVIPG -KWEG NG--- ----L LGIHI S 100
                   : :   :::        : : ::..:    ::::..: *.     .:          **: :

RnGRASP55       FCS FDGAN ENVWH VLEVES NSPAA LAGLR PHSDY IIG-A DTVMN ESEDL FSLIE THEAK P 160
HsGRASP55       FCS FDGAN ENVWH VLEVES NSPAA LAGLR PHSDY IIG-A DTVMN ESEDL FSLIE THEAK P 160
TgGRASP         FSD VGNVM SEGVR VLSVAP NSPAA HAGLV ELEDW ILADS QGVFR DVDDL VDSVA AALNR H 180
PfGRASP         YEF LN-AL NEGVR ILEILE NSPAY QSQLI EYEDF IIGYD KGIFR NQDEF MSYIN MNNII K 159
                :  .. . .:  ::*.:   **** *  : *   .*:*:.   . : :.: : ::.. :

RnGRASP55       --- ------ ---LK LYVYNT DTDNC REVII TPNSA WGGEG SLGCG IGYGY LHRIP TRPFE E 209
HsGRASP55       --- ------ ---LK LYVYNT DTDNC REVII TPNSA WGGEG SLGCG IGYGY LHRIP TRPFE E 209
TgGRASP         --- ------ ---LQ IFVFNS STERI REVMV VPNNA WGGEG SLGCK LGSGY LHRLP ---FS R 226
PfGRASP         ENS HDKKV LFNTI LYVYNY KHENI RKVQI QLNDS WGGKG LLGCN VATGY LHKIP PCQIK D 219
                          ::*: * . :. *:*  :  * .:*** :* *** :. ****: :*    :.

RnGRASP55       GKK ISLPGQMTGT PITPLKDGFTQ VQLSSVSPP-SLSPPGTAGVEQ SLSGLSISSAPPAV 268
HsGRASP55       GKK ISLPGQMAGT PITPLKDGFTE VQLSSVNPP-SLSPPGTTGIEQ SLTGLSISSTPPAV 268
TgGRASP         RVR VTEAG AKGEE SLAPQAPSAADAD SSEPAPSGASDAVGSVAVGSAAAAQAR SSTQSES 286
PfGRASP         KEE KENIN LKESFSSSHI KNNESESNNYKKEH MDITK VNDLE IVLLN DSVGKNTNN INNT 279
                   .    .     . :    . :: :  .       .  .   :    :    :.

RnGRASP55       SNVLSTGVPTVPLLPPQVNQS----LASVPPMNPAATLPSLMPLSAGLPNLPNLPSLSNF 324
HsGRASP55       SSVLSTGVPTVPLLPPQVNQS----LTSVPPMNPATTLPGLMPLPAGLPNLPNL----NL 320
TgGRASP         VGH SVSPGEAVSAGRGNAA QS----EAR EEAFR VQKEE GKQAT GDRED KEIYIL DSDGAP 342
PfGRASP         ESNEKNSTNIIPLWKNEAE KSGREDKNY INSQDVELNH DNNIK YMKND SNSNSHSSNGSS 339
                 .    .     :.    :. :*          .                  :

RnGRASP55       NLPAPHIMPGVGLP----ELG KPGLPPLPSLPPRNVPGIAPLPMPSDFLPS---FPLVPE 377
HsGRASP55       NLPAPHIMPGVGLP----ELV NPGLPPLPSMPPRNLPGIAPLPLPSEFLPS---FPLVPE 373
TgGRASP         VLLTPSIEFESAYPPYAHALEE EDVWSVGAAED SDFPACPDELDRPNFEFQTRNYCLY PV 402
PfGRASP         KTLEVDMD NNAET INYDDK FSYELKSTLDDQPEE DMHEI KKENF QNEQV ILEDI KREE PN 399
                       :          :      . :       :.          :           *

RnGRASP55       GSSAASAGEPLSSLP------A MG-PPSDPVMTTAKA DTSS-LTVDVM SPASKVPTTVED 429
HsGRASP55       SSSAASSGELLSSLP------- PTSNAPSDPATTTAKA DAASSLTVDVT PPTAKAPTTVED 427
TgGRASP         TNSPLKEALPLCSDR----- -EVRQ GDSDRGSDGASPAAYASPYFAYDF PYAAAMAR HTS 456
PfGRASP         NSQEIKNEI DTYNF KNEDI KDEIH IIKPEPINTDNDK TSRLVESYSDYVKKMT IYSKEMN 459
                 ..  .       .                .:       .  :                 :   .

RnGRASP55       RVSDCTPAMEKPVSAVTDANA SGAS------ ----- ----- ----- ----- ------ -- 454
HsGRASP55       RVG DSTPVSEKPVSAAVDANA SESP------ ----- ----- ----- ----- ------ -- 452
TgGRASP         AVF TGPPRPGEVVSIPSLH---- ----- ----- ----- ----- ------ ----- ----- 475
PfGRASP         EIY ESMNKNSEILNSIKMNATF NYVND NNNNN NNNVL NVDKR VNTEEY KNVDSQEIPILI 519
                 :         : :.

RnGRASP55       --- ----- ----- ------ ----- ----- ----- ----- ----- ----- -----
HsGRASP55       --- ----- ----- ------ ----- ----- ----- ----- ----- ----- -----
TgGRASP         --- ----- ----- ------ ----- ----- ----- ----- ----- ----- -----
PfGRASP         NPYENTTIL NSSKMDNITK GTYIN NKNDD IYSNENVQHF HEPSFNYVHDV RKNI  573
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Fig. 1. Structure of *Pf*GRASP.
(A) Schematic of the putative domain structure of *Pf*GRASP, which comprises a N-terminal myristoylation motif (red), a well-conserved N-terminal GRASP domain (grey) and an unconserved C-terminus implicated in phosphorylation (blue, P-domain). (B) Comparison of *Pf*GRASP with rat GRASP55 (*Rn*GRASP55; GenBank AF110267), human GRASP55 (*Hs*GRASP55; GenBank AAH07770) and *Toxoplasma gondii* GRASP55 (*Tg*GRASP, ToxoDB TgTwinScan_6910). Asterisks indicate identical residues and colons indicate conserved residues. Proline and serine residues are highlighted in blue in the conserved P-domain; a cross-species conserved glycine is red.

because of tandem repeat insertions and/or homopolymer runs) are well known in *P. falciparum*, the biological significance is unknown (Gardner et al., 1998; Pizzi and Frontali, 2000; Brocchieri, 2001). In the mammalian system this poorly conserved C-terminus is characterised by serine/proline richness (e.g. 33% in human GRASP55) and is a known substrate for mitotic phosphorylation (Shorter and Warren, 2002; Wang et al., 2005). By contrast, the C-terminal domain of *Pf*GRASP is only slightly enriched in serine and proline (12%). Nevertheless the C-terminal domain does encompass 20 putative serine phosphorylation sites (NetPhos 2.0 server) (Blom et al., 1999).

*Pf*GRASP is transcribed and expressed throughout the asexual life cycle

Conclusive microarray and proteomic data on *Pfgrasp* is not available (Bozdech et al., 2003; Le Roch et al., 2003; Florens et al., 2002). Therefore, we investigated *Pfgrasp* transcription and expression throughout the asexual life cycle. *Pfgrasp* gene transcription was analysed by real-time RT-PCR throughout the asexual blood stages of the parasite. A stage-specific control was performed using the early-transcribed gene *etramp* (Spielmann et al., 2003). Although transcription of *etramp* peaks in early stages (compared with levels of *actin* transcription), *Pfgrasp* is transcribed broadly across the asexual life cycle (Fig. 2A). This is reflected in the protein expression pattern of *Pf*GRASP. Stage-specific immunoblots using *Pf*GRASP-specific antibodies on synchronised parasite pellets show a major band at 70 kDa throughout the asexual life cycle (apparent M_r of *Pf*GRASP is 69×10^3, Fig. 2B).

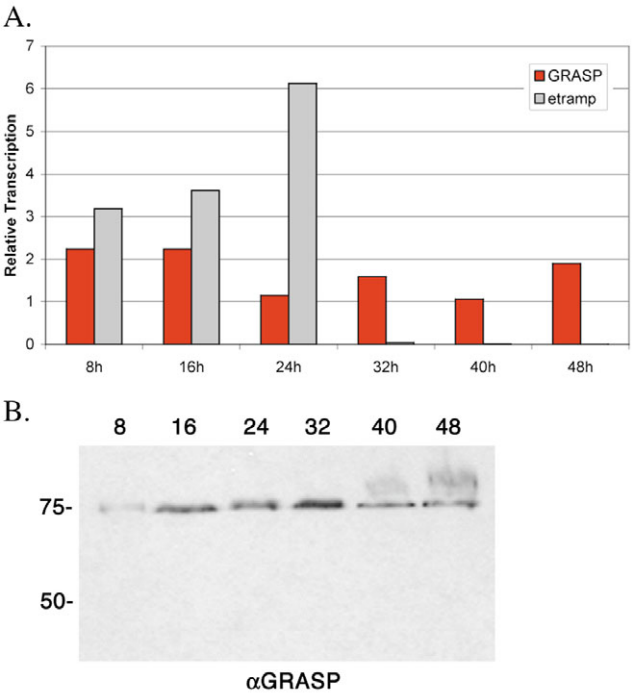

Fig. 2. Expression of *Pf*GRASP in the asexual blood stages. (A) Transcription of *Pfgrasp* (red) was analysed by real-time RT-PCR using total RNA extracted from tightly synchronised parasites every 8 hours. A stage-specific control was performed using the early transcribed *etramp* gene (grey). Relative gene expression is shown in bar graphs. This ratio was calculated by comparing the average transcription of *Pfgrasp* and *etramp* with transcription of the housekeeping gene *actin*, which was set to 1. Relative quantification through real-time RT-PCR showed no stage-specific gene regulation for *Pfgrasp*. (B) Immunoblot analysis of wild-type parasites (3D7). Proteins from synchronised parasite cultures from samples taken every 8 hours, were separated by SDS-PAGE on a 10% gel under reducing conditions. Approximately equal amounts of parasite protein were loaded. Using anti-*Pf*GRASP-specific antibodies, one major 70 kDa band can be detected throughout the asexual life cycle. Positions of molecular size markers (in kDa) are indicated.

*Pf*GRASP-GFP-expressing parasites and subcellular localisation

To morphologically characterise the Golgi in live parasites, a transgenic cell line expressing the intrinsic Golgi marker *Pf*GRASP as a chimeric protein with GFP was generated. Full-length *Pf*GRASP was fused to GFP and transfected into parasites. To confirm expression of the fusion protein, western blot analysis with anti-GFP antibodies were performed on transgenic parasites. A band corresponding to *Pf*GRASP-GFP of ~100 kDa was detected in the transgenic cell line but not in wild-type (WT) parasites (Fig. 3A, apparent M_r of *Pf*GRASP-GFP is 97×10^3). Additionally, the antibodies recognized two smaller proteins, presumably GFP breakdown products previously described for GFP-expressing parasites (Waller et al., 2000). Antibodies specific to *Pf*GRASP recognized the endogenous protein of ~70 kDa in both the WT and transgenic cell line. In the transgenic parasite line an additional band of

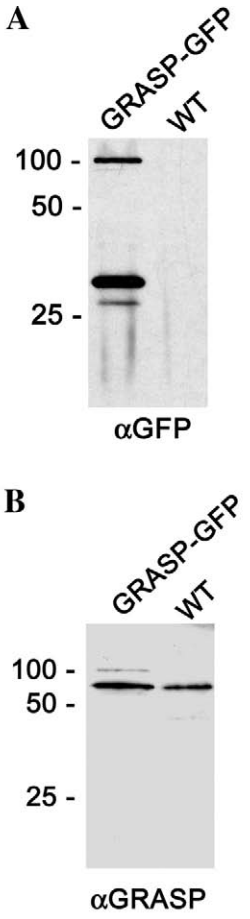

Fig. 3. Expression of *Pf*GRASP-GFP in transgenic parasites. (A) Immunoblot using GFP-specific antibodies on wild-type (WT) and *Pf*GRASP-GFP expressing parasites (GRASP-GFP). A band of ~100 kDa, representing the GFP-fusion protein, is recognized by GFP-specific antibodies in the transgenic, but not in the WT parasite line. In addition, two smaller bands, possibly GFP breakdown products can be detected. (B) Anti-*Pf*GRASP-specific antibodies recognize an ~100 kDa *Pf*GRASP-GFP fusion protein in addition to the endogenous *Pf*GRASP protein of 70 kDa in *Pf*GRASP-GFP-expressing parasites.

100 kDa corresponding to the *Pf*GRASP-GFP fusion protein was detected (Fig. 3B).

We investigated the localisation of *Pf*GRASP-GFP by either fluorescence microscopy of live cells expressing a green fluorescent fusion protein or indirect immunofluorescence of fixed parasites. In trophozoites (<24 hours post invasion) *Pf*GRASP-GFP was localised to two tightly defined compartments within the parasite juxtaposed to the nucleus with some cytoplasmic background fluorescence (Fig. 4A and supplementary material Movie 1). Importantly, *Pf*GRASP specific antibodies used on either WT or *Pf*GRASP-GFP expressing parasites reveal a similar staining pattern (Fig. 4B and Fig. 5A). Low levels of unspecific staining can be detected and may be a result of fixation.

Colocalisation of *Pf*GRASP-GFP with ER and Golgi marker proteins

To establish *Pf*GRASP-GFP as a marker for the Golgi and to generate more data with respect to Golgi architecture in *Plasmodium* we performed immunofluorescence assays using specific antibodies against previously described marker proteins for the ER and Golgi. Specifically, we used antibodies against the ER marker BiP (Elmendorf and Haldar, 1993a), the cis-Golgi marker ERD2 (Elmendorf and Haldar, 1993a; Van Wye et al., 1996) and the trans-Golgi marker Rab6 (De Castro et al., 1996; Van Wye et al., 1996).

ERD2 (ER-retention-defective complementation group 2), a seven-transmembrane-spanning receptor (Semenza et al., 1990; Lewis et al., 1990; Pelham et al., 1993) recognizes a C-terminal motif of X-aspartate-glutamate-leucine (XDEL) on certain soluble ER proteins and retrieves those proteins that have leaked to the Golgi complex back to the ER (Wilson et al., 1993; Lewis and Pelham, 1992). It is used as a cis-Golgi marker and can be localised to a single site adjacent to the nucleus in early (10 hours post-invasion) stages of the parasite, whereas in trophozoites two foci of *Pf*ERD2 are present (Elmendorf and Haldar, 1993a). Immunofluorescence assays with anti-*Pf*ERD2 antibodies show colocalisation to *Pf*GRASP-GFP suggesting identity of these cellular compartments (Fig. 5B). Using antibodies directed against the ER marker protein *Pf*BiP the ER could be visualised in young parasites as a ring of fluorescence encircling the nucleus of the parasite (Fig. 5C and supplementary material Movie 2). Parts of this membranous system, which are reminiscent of the tip of two protrusions extending from the nuclear envelope described recently for a *Pf*BiP-GFP fusion in *Plasmodium* (Van Dooren et al., 2005), are in close proximity to the *Pf*GRASP-GFP-defined compartment. Altogether, the close association of parts of the ER with the *Pf*GRASP-defined compartment and the colocalisation of ERD2 validates *Pf*GRASP as a Golgi marker.

Similar studies were performed using anti-*Pf*Rab6 antibodies. Rab6 are small GTPases used as markers of trans-Golgi compartments in various systems including *Plasmodium* (De Castro et al., 1996). Immunofluorescence patterns of early parasite stages (<24 hours post invasion) resulted in several (1-4) distinct fluorescent sites different to those defined by *Pf*GRASP (Fig. 5D). In some cells partial association of *Pf*Rab6 with the *Pf*GRASP-defined compartments could be observed (supplementary material Movie 3). This suggests that the trans-Golgi topology is distinct from those defined by *Pf*GRASP.

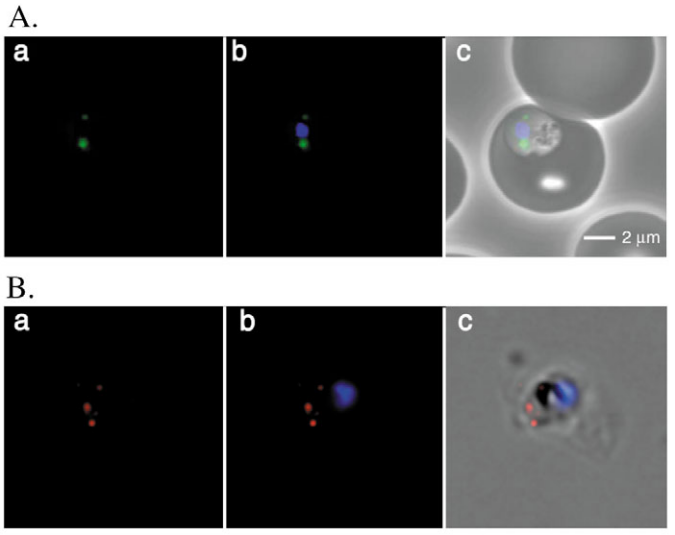

Fig. 4. Localisation of *Pf*GRASP by confocal and fluorescence microscopy in trophozoites (<24 hours post invasion). (A) Full-length *Pf*GRASP is expressed as a GFP-fusion protein. Using fluorescence of the GFP reporter protein in live cells, *Pf*GRASP-GFP distribution (green) is restricted to two compartments within the parasite (a) (see also supplementary material Movie 1). These compartments are in close proximity to the nucleus (b, blue). Merge with bright-field image (c). (B) Fixed WT parasites were incubated with *Pf*GRASP-specific antibodies. *Pf*GRASP-specific antibodies (a, red) show a similar fluorescence pattern in fixed cells compared with *Pf*GRASP-GFP expressing parasites. However, additional unspecific staining can be detected. Merge with DNA-specific stain (b, blue). Merge with bright-field image (c). Bar, 2 μm.

Golgi dynamics during the asexual life cycle of *Plasmodium*

The Golgi is multiplied and distributed during mitosis and cellular division. Schizogony of *P. falciparum* requires duplication as well as multiplication of the Golgi within 48 hours. We used *Pf*GRASP-GFP expressing parasites to visualise Golgi dynamics throughout the asexual life cycle of *Plasmodium*. In early parasite stages (8-16 hours post invasion) one single oval Golgi compartment in close proximity to the nucleus can be observed (Fig. 6a-b). As the parasite develops, but prior to nuclear division, a second Golgi is formed (24 hours post invasion, Fig. 6c). As nuclear division commences (32 hours), further Golgi multiplication occurs (Fig. 6d). This results in multiple Golgi compartments ensuring that each merozoite inherits one Golgi (Fig. 6e-g). These results were confirmed using a second transgenic cell line expressing the cis-Golgi marker ERD2 as a GFP fusion protein (supplementary material Figs S1 and S2). It is interesting to note that the expression of *Pf*ERD2-GFP also results in additional perinuclear staining reminiscent of the ER. This finding agrees with previous work showing the presence of low levels of *Pf*ERD2 in the ER (Van Wye et al., 1996). Owing to the additional perinuclear staining, (parts of) the ER can be visualised and followed during schizogony (supplementary material, Fig. S2).

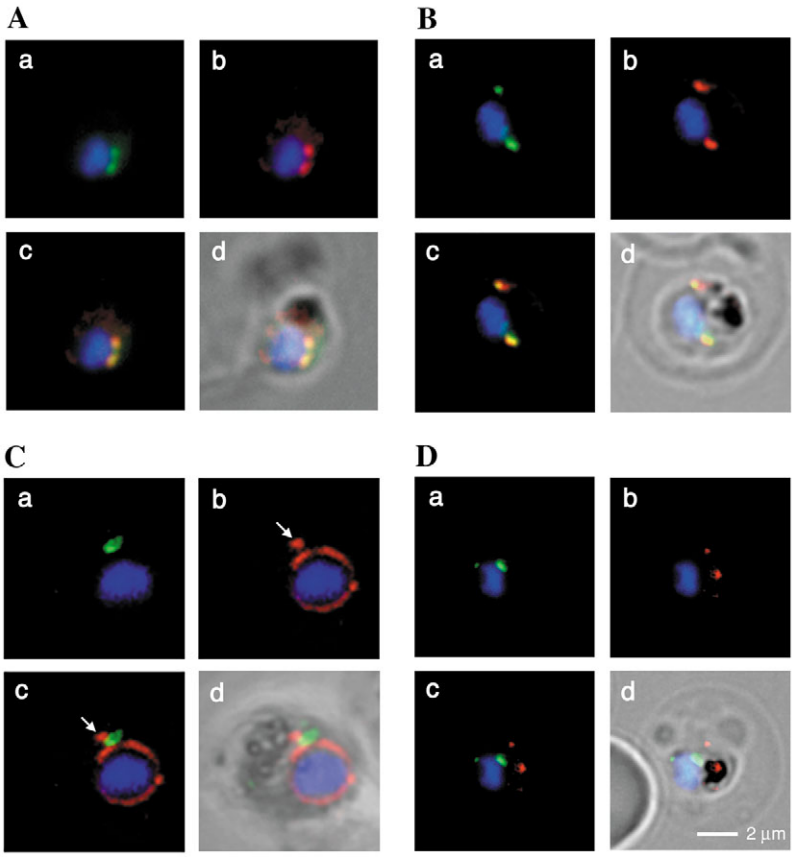

Fig. 5. Spatial organisation of the *Pf*GRASP-GFP defined compartment by fluorescence microscopy on fixed parasites. (A) *Pf*GRASP-GFP colocalises with anti*Pf*GRASP-specific antibodies. *Pf*GRASP-GFP is tightly confined to two compartments (a, green) near the parasite nucleus (a, blue). Anti*Pf*GRASP-specific antibodies show a similar staining pattern (b, red with nucleus in blue). Merged image shows the colocalisation of the compartments defined by either *Pf*GRASP-specific antibodies or *Pf*GRASP-GFP-expressing parasites (c, yellow). (B) *Pf*GRASP-GFP colocalises with the cis-Golgi marker ERD2. *Pf*GRASP-GFP (a, green) accumulates in two discrete compartments in close proximity to the nucleus (a, blue). Anti-*Pf*ERD2 antibodies recognize similar structures (b, red with nucleus in blue). Merged image shows colocalisation of compartments (c, yellow). (C) *Pf*GRASP-GFP defines a compartment that is distinct from the ER (see also supplementary material Movie 2). At the early stages of the parasite life cycle (<16 hours post invasion) *Pf*GRASP is restricted to one compartment (a, green) juxtapose to the nucleus (a, blue). The ER is visualised by anti-*Pf*BiP-specific antibodies (b, red). The membranous system of the ER forms an envelope around the nucleus (b, blue) with one protrusion (indicated by arrow). Merged image shows no colocalisation of the two compartments (c). (D) *Pf*GRASP-GFP does not colocalise with the trans-Golgi marker *Pf*Rab6. *Pf*GRASP accumulates in two discrete foci (a, green) adjacent to the nucleus (a, blue). Antibodies against *Pf*Rab6 visualise two distinct sites within the parasite (b, red with nucleus in blue). Merged image shows no colocalisation of the *Pf*GRASP defined compartment with *Pf*Rab6 (c) (see also supplementary material Movie 3). All panels labelled d in A-D are merges of fluorescent and bright-field images. Bar, 2 μm.

Subcellular localisation of *Pf*GRASP depends on an N-terminal putative myristoylation motif

Golgi localisation of GRASP proteins requires N-terminal myristoylation (Barr et al., 1997; Barr et al., 1998; Shorter et al., 1999). The covalent attachment of fatty acid chains facilitates localisation of water-soluble proteins to a membrane after its synthesis in the cytosol. This modification involves the attachment of myristic acid via an amide linkage to an N-terminal glycine. Phylogenetic analysis suggests this myristoylation site is conserved in *Pf*GRASP (Fig. 1). To determine if targeting of *Pf*GRASP depends on the putative N-terminal myristoylation site, this motif was disrupted by point mutation. A GFP-fusion construct was generated where the amino acid glycine was replaced by alanine at position 2 of the protein sequence (*Pf*GRASP-G_2/A-GFP) and transfected into parasites. Perturbation of the putative N-terminal myristoylation site resulted in the accumulation of the protein in the cytoplasm of the parasite (Fig. 7). This data suggests that targeting of *Pf*GRASP requires glycine at position 2 presumably because it serves as a substrate for the N-myristoyltransferase (PlasmoDB PF14_0127) (Gunaratne et al., 2000) linking myristic acid via an amino bridge to the protein backbone and thereby facilitating membrane attachment.

*Pf*GRASP distribution is insensitive to BFA treatment

Brefeldin A (BFA) is a fungal metabolite that specifically inhibits anterograde transport from ER to Golgi compartments (Lippincott-Schwartz et al., 1989; Orci et al., 1991). It allows fractionation of Golgi proteins. Proteins like ERD2 or N-acetylmannosidasetransferase are relocated to the ER upon BFA treatment (Elmendorf and Halder, 1993a; Pelletier et al., 2002). By contrast, structural Golgi components like the GRASP protein family are insensitive to BFA (Nakumura et al., 1997; Pelletier et al., 2002; Seemann et al., 2002). After BFA treatment of *Pf*GRASP-GFP expressing parasites, we observed no relocation of the fusion protein to the ER (Fig. 8).

Discussion

A GRASP homologue in *P. falciparum*

In recent years, Golgi matrix proteins have been characterised that define structure and identity of the Golgi complex (Short and Barr, 2000). GRASP proteins are implicated in cisternal stacking and signalling (Barr et al., 1997; Shorter at al., 1999; Seeman et al., 2000) and have been used for investigating Golgi development and architecture in protozoa (Pelletier et al., 2002; He et al., 2004). GRASP proteins interact with the golgin family of coiled-coil proteins providing an exoskeleton for this organelle. To date two GRASP paralogues are described in the mammalian system: the cis-Golgi marker GRASP65 interacting with GM130 and medial-Golgi marker GRASP55 interacting with Golgin45 (Barr et al., 1997; Barr et al., 2001). We identified a single *grasp* homologue in the genome of *P.*

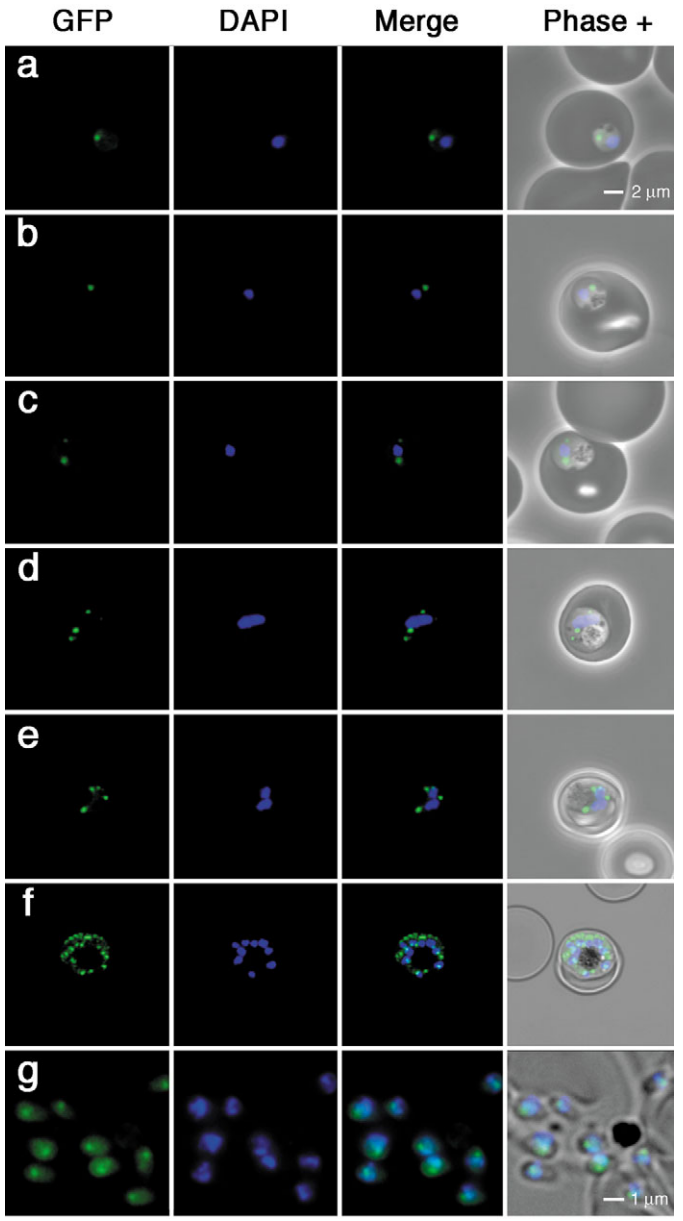

Fig. 6. Golgi dynamics throughout the asexual life cycle of *Plasmodium*. Live images of transgenic parasites expressing *Pf*GRASP-GFP were visualised by confocal microscopy. (a-b) Images 8-16 hours post invasion. In ring-stage parasites *Pf*GRASP-GFP is restricted to one compartment in close proximity to the nucleus (blue). (c) Images 24 hours post invasion. A second Golgi is generated prior to nuclear division. (d-e) Cells 32-40 hours post invasion. As the parasite matures, nuclear division commences and is accompanied by multiplication of the Golgi. (f) Cells 46 hours post invasion. The parasite has nearly reached the final stage of schizogony where each forming merozoite will be equipped with one Golgi and nucleus. (g) Released parasites at 0 hours. Each merozoite has inherited one Golgi. Bar, 2 μm (a-f); 1 μm (g).

falciparum, established its expression profile and used this putative Golgi matrix protein to analyse Golgi organisation and dynamics during the asexual life cycle of the parasite. The homologue of GRASP in *P. falciparum* has an apparent M_r of 69×10^3 and is slightly more similar to mammalian GRASP55 than GRASP65 (34% vs 30% similarity). *Pf*GRASP has a conserved domain structure compared with known GRASP proteins: (1) a N-terminal myristoylation motive; (2) a highly preserved N-terminal GRASP domain known to be both necessary and sufficient for dimerisation and trans oligomerisation (Wang et al., 2005); (3) a short middle so-called GM130 binding domain; and (4) a highly divergent C-terminus. The proline/serine-rich C-terminus of mammalian GRASP proteins is implicated in mitotic regulation via phosphorylation and is a substrate of caspases during apoptosis (Wang et al., 2005; Lane et al., 2002). Putative mitotic kinases like Cdc2-related enzymes are present and characterised in the parasite (Kappes et al., 1999; Le Roch et al., 2000) and might recognize phosphorylation sites in the C-terminal domain of *Pf*GRASP. The region currently known to be important for GM130 binding is conserved in GRASP65 and GRASP55 and could be mapped to $_{194}$GYGXXHRI$_{201}$ (Barr et al., 1998), although binding of GM130 to GRASP55 might not occur in vivo (Shorter et al., 1999). It is interesting to note that this highly conserved region in mammals and yeast is altered in *P. falciparum* to $_{205}$AYGXXHKI$_{212}$ (Fig. 1) suggesting differences in the *Pf*GRASP-interacting protein complex. Coincidentally, only coiled-coil proteins with very low similarity to GM130 and Golgin45 could be identified in the genome of the parasite.

Defining the Golgi

Cell lines expressing GFP fusion proteins allow localisation and tracking of the tagged protein in live cells during the cell cycle. This transgenic approach circumvents fixation of the cells and therefore might prevent ultrastructural disturbance as exemplified recently (Van Dooren et al., 2005). Using fluorescence microscopy on GFP-tagged *Pf*GRASP we

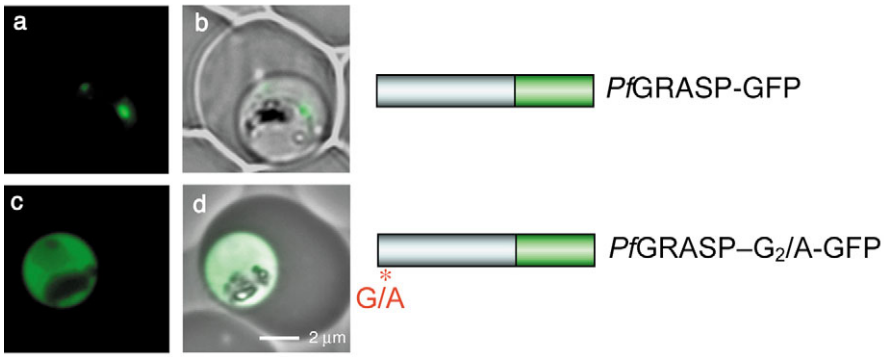

Fig. 7. Localisation of *Pf*GRASP depends on a functional N-terminal myristoylation motif. Fluorescence microscopy was performed on live parasites. (a) In parasites expressing *Pf*GRASP-GFP the protein is restricted to two tightly defined compartments (green). (b) Merge with bright-field image. (c) Mutation of the putative N-terminal myristoylation site (from glycine to alanine) abolishes targeting of *Pf*GRASP-GFP and results in a cytoplasmic distribution of the fusion protein. (d) Merge with bright-field image. Bar, 2 μm.

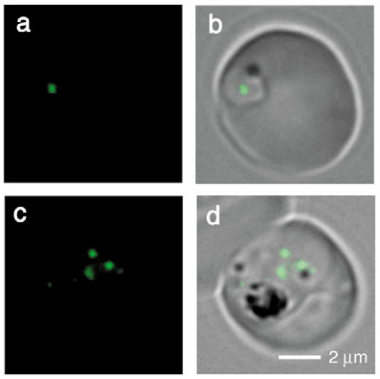

Fig. 8. Effect of Brefeldin A on the distribution of *Pf*GRASP. (a) Parasites were incubated with BFA for 24 hours. Localisation of *Pf*GRASP-GFP in live parasites is focused to one compartment within the parasite (green). (b) Merge with bright-field image. (c) As a control, cultures were incubated with ethanol to ensure normal growth and morphology. The image displays a mature parasite (>32 hours) with multiple fluorescing foci (green). (d) Merge with bright-field image. Bar, 2 μm.

localised the protein within distinct foci in the parasite. Minor levels of background fluorescence (supplementary material Movie 1) point to a cytoplasmic pool of *Pf*GRASP-GFP as described previously for mammalian GRASP65 (Ward et al., 2001). It was suggested that GRASP65 shuttles between a cytoplasmic pool and Golgi membrane association. Up to 30% of the endogenous GRASP65 (and up to 55% of GRASP65-GFP in the transgenic cell line) was distributed in the cytoplasm (Ward et al., 2001).

We validated the subcellular localisation of the fusion protein by using *Pf*GRASP specific antibodies (Fig. 5A) and established *Pf*GRASP as a Golgi marker. This is the first time that the Golgi apparatus in *Plasmodium* has been visualised in live cells. To further analyse the extent of Golgi compartmentalisation, the ER marker BiP, the cis-Golgi marker ERD2 and the trans-Golgi marker Rab6 were used in colocalisation experiments. Firstly, we analysed the spatial relationship between the ER and the *Pf*GRASP-defined compartments by using *Pf*BiP specific antibodies. We show that the ER, which forms a perinuclear envelop with 1-2 protrusions (>24 hours post invasion), lies in close proximity to the *Pf*GRASP-defined compartment (Fig. 5C and supplementary material Movie 2). These ER protrusions might reflect ER exit sites and it will be interesting to further analyse the functional relationship between the ER exit sites and the Golgi.

ERD2 recognizes a C-terminal XDEL motif on certain soluble ER proteins in the cis-Golgi and retrieves them into the ER (Wilson et al., 1993; Lewis and Pelham, 1992). ERD2 was used as a cis-Golgi marker in *Plasmodium* and was localised to defined loci adjacent to the nucleus (Elemendorf and Haldar, 1993a). Further, these antibodies were used in immunofluorescence assays to analyse ERD2 distribution during schizogony, showing a close spatial relation between forming nuclei and ERD2 distribution in *P. yoelii* (Noe et al., 2000). Using the fluorescence of *Pf*GRASP-GFP and anti-*Pf*ERD2-specific antibodies we showed that *Pf*GRASP colocalises with the cis-Golgi marker ERD2 (Fig. 5B). As *Pf*GRASP appears to be more homologous to the mammalian medial-Golgi marker GRASP55, this colocalisation could argue for a limited compartmentalisation of the Golgi. It could also indicate that *Pf*GRASP might be a functional equivalent of the cis-Golgi marker GRASP65 (therefore colocalising with ERD2) and the medial-Golgi is defined by an as yet unidentified *Pf*GRASP homologue. In the absence of an appropriate medial-Golgi marker a conclusive answer remains elusive.

Another membrane-associated protein, the small GTPase *Pf*Rab6, was used to analyse Golgi architecture in the malaria parasite. Rab proteins are small, GTP-binding proteins that play a pivotal role in the regulation of vesicular trafficking in eukaryotic cells (Ward et al., 1997). Parasite-specific *Pf*Rab6 antibodies were used in indirect fluorescence microscopy assays and showed a localisation distinct from ERD2 in early stages (Van Wye et al., 1996). This is supported by our data showing a differential distribution of the *Pf*GRASP-GFP-defined compartment and *Pf*Rab6 and in some cases some loose association (Fig. 5D and supplementary material Movie 3). This differential distribution was used as an indication for a functional but primitive and unstacked Golgi with distinct compartments in early stages of the parasite (Van Wye et al., 1996). Another explanation for the loose association of Rab6 and the Golgi might be the multifunctional role of Rab6 in vesicular transport. In the mammalian system it could be shown that Rab6 (with its two isoforms) does not only function in intra-Golgi transport (Martinez et al., 1994) but is also required for protein recycling between endosomes and the trans-Golgi network (Mallard et al., 2002; Stedman et al., 2003) and even to the ER (White et al., 1999). In yeast, the Rab6 homologue Ypt6p has also been implicated in multiple roles, for example the retrieval of proteins from endosomes to the trans-Golgi network, intra-Golgi retrograde and Golgi to ER transport (Siniossoglou et al., 2001; Bensen et al., 2001; Luo and Gallwitz, 2003). Therefore, the precise function of Rab6 and its associated compartments in the parasite have to be further investigated.

We could show that *Pf*GRASP remains restricted within the parasite boundaries throughout the asexual life cycle, as shown previously for *Pf*ERD2 (Fig. 6, supplementary material Fig. S2) (Elemendorf and Haldar, 1993a; Noe et al., 2000). An interesting observation by other groups is the localisation of the Golgi markers sphingomyelin synthase and two homologues of the COPII protein complex *Pf*Sar1p, *Pf*Sec31p beyond the parasite boundaries in the erythrocyte cytosol and membranes (Elmendorf and Haldar, 1994; Albano et al., 1999; Adisa et al., 2001; Adisa et al., 2002). This differential localisation (compared with *Pf*ERD2) was used to argue for a rather unusual Golgi organisation in *Plasmodium*, which at least functionally expands into the host cell cytoplasm. In support of this theory it was shown that the export of *Pf*Sar1p and *Pf*Sec31p to the erythrocyte cytosol was inhibited by BFA treatment (Adisa et al., 2002). It will be interesting to further investigate the putative export of COPII proteins and vesicles to the host cell cytosol and to analyse the spatial relationship between Golgi and COPII vesicles.

*Pf*GRASP depends on a functional myristoylation site at the N-terminus of the protein. We produced a cytoplasmic variant of *Pf*GRASP-GFP by substituting glycine at position 2 of the amino acid sequence to alanine (Fig. 7). This suggests that

*Pf*GRASP, like its mammalian counterparts, is a substrate for N-myristoylation (Barr et al., 1997; Wang et al., 2003). Additional palmitoylation could also play an enhancing role in membrane anchoring of *Pf*GRASP as reported for GRASP55 (Kuo et al., 2000).

Brefeldin A (BFA), a fungal metabolite, is an important tool to study protein trafficking in the endomembrane system (Lippincott-Schwartz et al., 1989; Lippincott-Schwartz et al., 1990). It inhibits secretion of vacuolar protein transport, specifically blocking secretory export to post-Golgi compartments and has been shown to reorganise the Golgi apparatus (Tamaki and Yamashina, 2002; Nebenfuhr et al., 2002). BFA relocates Golgi enzymes like N-acetylglucosaminotransferase (NAGTI) to the nuclear envelope but does not effect distribution of Golgi matrix proteins like GRASP (Nakamura et al., 1997; Pelletier et al., 2002; Seemann et al., 2002). BFA has been used extensively to investigate protein trafficking in *Plasmodium,* and it was shown that the cis-Golgi marker ERD2 is redistributed to the ER upon BFA treatment (Elemendorf and Haldar et al., 1993; Wickham et al., 2001). In contrast to ERD2, *Pf*GRASP distribution in *Plasmodium* is not affected, stressing its function as a Golgi exoskeleton matrix protein. This also implies that Golgi proteins in *Plasmodium* can be fractionated upon BFA treatment.

Biogenesis of the Golgi

Golgi multiplication in *T. gondii* and *T. brucei* has been analysed in great detail using video microscopy. In *T. gondii* the new Golgi grows by lateral extension followed by medial fission (Pelletier et al., 2002). In *T. brucei* the Golgi appears de novo and was shown to be rather an independent entity (He et al., 2004). We used the *Pf*GRASP-GFP expressing cell line to follow Golgi biogenesis in *Plasmodium* monitoring parasites throughout the asexual life cycle because single-cell video microscopy over longer time periods is not yet technically feasible in *P. falciparum*. Nevertheless, our data indicates that Golgi duplication starts between 16 and 24 hours post-invasion prior to nucleus division, matching the increased metabolic activity of the parasite. After schizogony is completed each merozoite displays one single Golgi (Fig. 6g). It is important to note that our data do not exclude the possibility of an excess Golgi during cell division as described for *Toxoplasma* and *Trypanosoma*. Most of these additional Golgi disappear before the mother cell undergoes cytokinesis (Pelletier et al., 2002; He et al., 2004). Owing to technical restrictions our data does not allow a conclusive prediction of the assembly of new Golgi cisternae in the parasite. The new Golgi might be formed de novo in close contact with the ER export sites or by fission of the old Golgi. In fact, both mechanisms could be operational in the biogenesis of a new Golgi as proposed for *Pichia pastoris* (Bevis et al., 2002).

Form follows function?

The biosynthetic-secretory pathway leads outwards from the ER towards the Golgi and the cell surface with the Golgi apparatus as the central hub (Warren and Malhotra, 1998). The major function of the Golgi is thought to be the processing and sorting of newly synthesised proteins and lipids. The ordered architecture of this organelle allows a functional compartmentalisation. In the mammalian system, two GRASP proteins, GRASP55 and GRASP65, are implicated in Golgi membrane stacking. GRASP65 is located in cis-Golgi membranes whereas GRASP55 is located more towards the medial Golgi stack. This differential localisation was used to argue that the GRASP protein family helps determine stacking of different cisternal layers (Wang et al., 2003). It was suggested that organisms with only one *grasp* homologue (e.g. *Saccharomyces cerevisiae* and *T. brucei*) might possess a simpler Golgi organisation compared with the mammalian cell system (He et al., 2004). In the *Plasmodium* genome only one *grasp* homologue could be identified. This simple architecture might be mirrored in a reduced range of biochemical processes taking place in this compartment. For instance, the *Plasmodium* genome encodes only a cryptic glycosylation capacity with no O-linked glycosylation pathway and reduced N-glycosylation (Templeton et al., 2004). In this light, a high degree of compartmentalisation of the Golgi to allow a spatial as well as biochemical separation of glycoprocessing seems to be unnecessary.

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