Plasmodium falciparum secretory pathway: Characterization of PfStx1, a plasma membrane Qa-SNARE

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Abstract

SNAREs (soluble N-ethylmaleimide-sensitive factor attachment protein receptors) play a central role in regulating and facilitating vesicular traffic in eukaryotic cells. While SNAREs have been well characterized in other eukaryotes, little is known about their role in the unique protein trafficking pathways in *Plasmodium falciparum*. We have identified seven Qa-SNAREs in the *P. falciparum* genome and confirmed the gene structure of all seven, which in one case differs from the predicted structure in the database. Based on comprehensive sequence alignments we made predictions for the intracellular locations of all seven *P. falciparum* Qa-SNAREs, and confirmed the predicted location for one Qa-SNARE, PfStx1, which is most closely related to other eukaryotic plasma membrane Qa-SNAREs such as syntaxin 1. This is the first identified trafficking component localized proximal to the *P. falciparum* plasma membrane.

Plasmodium species are a diverse and extremely successful group of intracellular parasites which in humans cause 300–500 million cases and more than 1 million deaths from malaria each year [1]. The majority of human malaria mortality is caused by *Plasmodium falciparum*, which like all *Plasmodium* parasites has a complex life cycle involving both mosquito and human hosts. This complicated life cycle depends on a vast array of parasite-host interactions, and many of these interactions are controlled by the *Plasmodium falciparum* secretory pathway. For example during the intra-erythrocytic stage of *P. falciparum* development, which is the stage that causes all the symptoms and pathology of malaria, it is the secretory pathway that ingests hemoglobin from the erythrocyte cytosol to drive parasite growth and replication and it is the secretory pathways that traffics antigenically variable cytoadherence ligands to the erythrocyte plasma membrane to avoid the protective immune response.

Work on model organisms and cell lines has revealed a great deal about the molecular mechanisms of secretion, and both preliminary genome analysis and recent elegant mechanistic studies [2,3] have confirmed that many of these fundamental features of eukaryotic secretory pathways are conserved in *P. falciparum*. However, *P. falciparum* intra-erythrocytic stages

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also contain several unique organelles that are not readily classifiable into the classical eukaryotic secretory pathway. First, a food vacuole begins to form during the ring stage, where endocytosed hemoglobin is transported to and then metabolized [4,5]. Second, *P. falciparum* possesses an apicoplast, a non-photosynthetic plastid where fatty acids are synthesized and is unique to the phylum Apicomplexa [6,7]. Third, after invasion of the host erythrocyte, unique membrane-bound organelles called Maurer's clefts develop outside of the parasites own plasma membrane in the erythrocyte cytosol and appear to play a role in trafficking *P. falciparum* proteins to the erythrocyte plasma membrane [8]. It is currently not known how any of these organelles intersect with the classical eukaryotic secretory pathway.

Organelles maintain a distinct identity because protein transport to them is a tightly controlled event. Proteins are transported between organelles by in membrane-bound vesicles and the direction and specificity of vesicle transport is governed in large part by two families of proteins, SNAREs and Rabs. Rabs are small GTPases of the Ras superfamily that cycle between the cytosol (GDP bound) and organelle membranes (GTP bound) and Rab effector proteins aid in vesicle tethering as well as specificity of vesicle fusion [reviewed in 9]. Eleven Rab gene homologues have been identified in *P. falciparum* [10]. SNAREs are a family of typically membrane bound proteins that are all characterized by a relatively conserved coiled-coil SNARE domain near the C-terminus [11, reviewed in 12] and can be functionally classified as v-SNARES, which are present on vesicles, or t-SNARES, which are present on a target organelle. Another nomenclature divides the SNAREs into Q-SNAREs and R-SNAREs according to the presence of a glutamine or arginine residue at the core of the SNARE domain. Q-SNAREs are further subdivided into Qa-, Qb-, and Qc-SNAREs with Qa-SNAREs having homology to syntaxin 1a, the neuronal plasma membrane protein used to define the t-SNARE class [13]. Membrane fusion occurs when 3 Q-SNARE domains and one R-SNARE domain come together to form tetrameric helical bundle complex that drives the fusion between the vesicle and target compartment [14]. SNAREs have been found in the genomes of all eukaryotes studied to date from the primitive single celled *Giardia intestinalis* to *Homo sapiens* [15] and the published genome of *P. falciparum* contains numerous sequences with homology to SNARE domains. A preliminary analysis of such sequences identified 18 members of the SNARE family in *P. falciparum* [16], and noted several unusual features of these sequences, but did not classify such sequences into Qa-, Qb-, Qc- and R-SNARE sub-families.

Because of their role in specifying the fidelity of vesicle fusion and presence primarily on organelles rather than vesicles, Qa-SNAREs are of particular interest as organelle markers. Given the presence of several organelles of unknown provenance in *P. falciparum* intraerythrocytic stages, we were particularly interested in identifying and localizing *P. falciparum* Qa-SNAREs. To identify *P. falciparum* Qa-SNAREs in particular, rather than all genes encoding SNARE domains, we performed a comprehensive BLAST search analysis by querying the *P. falciparum* genome with the complete set of Qa-SNARE sequences from all major eukaryotic model organisms: *Saccharomyces cerevisiae, Caenorhabditis elegans, Drosophila melanogaster* and *Homo sapiens*. All significant hits (e value less than 0.05) were visually inspected for structural properties present in Qa-SNAREs from other species including size, lack of a signal sequence, a single transmembrane domain at the C-terminus, a coiled-coil region before the transmembrane domain with homology to the SNARE domain, and the presence of a second cluster of alpha helices at the N-terminus, the H_{abc} domain that distinguishes syntaxin homologs from other SNARE domain containing proteins.

This initial approach identified seven *P. falciparum* Qa-SNAREs, which are listed in Table 1 (these genes are listed as *PfStx* for their homology to the founding member of the Qa-SNARE class, syntaxin which has the gene name HsStx1a). The *P. falciparum* SNARE IDs previously assigned in a non Qa-SNARE specific manner are also included in Table 1 for comparison

[16]. RT-PCR confirmed that all seven genes are transcribed in blood stages (data not shown). Visual inspection of the sequence suggested that the exon/intron boundaries of two of the Qa-SNAREs, *PfStx4* and *PfStx6*, appeared to be possibly misannotated, with both genes having a particularly AT-rich stretch near the 5' end that could be introns rather than exons. We therefore cloned and sequenced the RT-PCR product for both genes. The *PfStx4* cDNA sequence was identical to the gene product predicted in PlasmoDB, confirming that this gene does consist of a single exon. By contrast, the cDNA product for *PfStx6* was smaller than the full-length genomic fragment (data not shown), confirming that *PfStx6* is a multi-exon gene, However, the 5' AT-rich region was missing from the *PfStx6* RT-PCR product, indicating that the predicted exon/intron boundaries of this gene are indeed incorrect. We used a combination of RT-PCR and 5'-RACE to sequence the full-length *PfStx6* transcript. This cDNA sequence showed that the PlasmoDB predicted *PfStx6* product in fact included a non-coding region as part of the first predicted exon, resulting in the prediction of a second transmembrane domain in *PfStx6,* which would have been unique among all eukaryotic SNAREs [16]. The actual *PfStx6* transcription start site begins 216bp down stream of the predicted start site, and our confirmed transcribed product includes several exon/intron boundaries that differ from the predicted product and encodes only one transmembrane domain near the C-terminus (GenBank Accession Number EF142857). This structure conforms much more closely to other eukaryotic Qa-SNAREs, including the presence of an N-terminal H_{abc} domain, confirming PfStx6 as a member of the *P. falciparum* Qa-SNARE family.

Having experimentally established the primary sequence of all seven *P. falciparum* Qa-SNAREs, we proceeded to sequence analysis. Figure 1a shows a CLUSTAL alignment of the seven *P. falciparum* Qa-SNAREs with their closest homolog among model organism Qa-SNAREs. Interestingly two of the *P. falciparum* Qa-SNAREs, PfStx1 and PfStx4, lack a conserved glutamate in the core of the SNARE domain (starred residue, Fig. 1a). However, the predicted plasma membrane Qa-SNARE sequences of several other protozoa, *Encephalitozoon cuniculi* and *G. intestinalis*, also lack this conserved glutamate indicating that it may not be universally conserved as once believed [17]. Because Qa-SNAREs that share the same intracellular location in different eukaryotes are often more closely conserved than are Qa-SNAREs in different locations in the same eukaryote, we were particularly interested in which Qa-SNAREs each Pf Qa-SNARE showed the highest homology to in their SNARE domain. In all seven cases, the SNARE domain of each *P. falciparum* Qa-SNARE was most closely related to one family of eukaryotic Qa-SNAREs (Fig 1a), allowing us to tentatively predict their intracellular location in *P. falciparum* parasites (Table 1).

PfStx1 was most closely related to plasma membrane Qa-SNAREs such as Syntaxin, down to the level of amino acid residues at the N-terminus (Fig 1b) that in Syntaxin interact with its binding partner, Munc-18 [18]. Published microarray data shows that *PfStx1* transcription is up-regulated in schizonts, which would be expected for a plasma membrane Qa-SNARE given that schizogony involves the creation of multiple new plasma membranes [19]. We therefore chose to investigate PfStx1 as a test case for the utility of predicting Qa-SNARE intracellular location based on sequence alignments. To avoid issues of mislocalization due to epitope tagging or over-expression, both of which have been known to cause mistargeting of Qa-SNAREs in other eukaryotes, we generated PfStx1-specific antisera to follow endogenous PfStx1. The PfStx1 cytoplasmic domain was expressed as a hexa-his tagged recombinant protein in *E. coli*, purified using affinity chromatography, and used to generate polyclonal antiserum. Probing total protein extracts from *P. falciparum* schizont stage parasites revealed a single band at 37kDa, in keeping with the predicted size of PfStx1 (Fig 2a). PfStx1 was not detectable in ring stage parasites using this antibody (data not shown), presumably due to the low expression level in this stage established by microarray profiling [19].

The anti-PfStx1 antiserum was then used in immunofluorescence experiments to determine the intracellular location of endogenous PfStx1. PfStx1 staining is visible at the periphery of early trophozoite stage parasites, consistent with a predicted plasma membrane location (Fig 2b). To confirm this, we performed co-staining in schizont stages. As expected, PfStx1 co-localized with PfMSP3 (Merozoite Surface Protein 3, a peripheral plasma membrane protein, Fig 2d) but is quite distinct from BiP, a marker for the *P. falciparum* endoplasmic reticulum (Fig 2c), as well as PfErd2, a marker for the cis-Golgi (data not shown). The experimentally established location of endogenous PfStx1, plasma membrane proximal, is therefore in agreement with the location predicted by sequence alignment with other eukaryotic Qa-SNAREs.

In conclusion, we have used RT-PCR and bioinformatics to annotate for the first time the Qa-SNARE family of *P. falciparum*, and established that PfStx1, which is most closely related to other plasma membrane Qa-SNARE, is localized proximal to the *P. falciparum* plasma membrane. This is only the second Qa-SNARE to be localized in *P. falciparum* and the first component of the *P. falciparum* plasma membrane vesicle trafficking machinery to be identified. Whether the Qa-SNARE predictions based on sequence alignment will hold true for the other *P. falciparum* Qa-SNAREs remains to be seen, but it should be pointed out that that the only other *P. falciparum* Qa-SNARE of known location, PfStx5, is in the cis-Golgi [16], which again conforms with the prediction based on sequence alignments (see Table 1). The prediction that PfStx2 is located in the trans-Golgi is perhaps the strongest, as the family of trans-Golgi Qa-SNAREs is one of the most conserved across the eukaryotic lineage. However, although the predicted locations of the other *P. falciparum* Qa-SNAREs are more tentative, given the interest in the mechanisms of protein trafficking to the food vacuole in particular, these predictions certainly provide obvious leads to follow, in this case PfStx3 and PfStx6.

Acknowledgments

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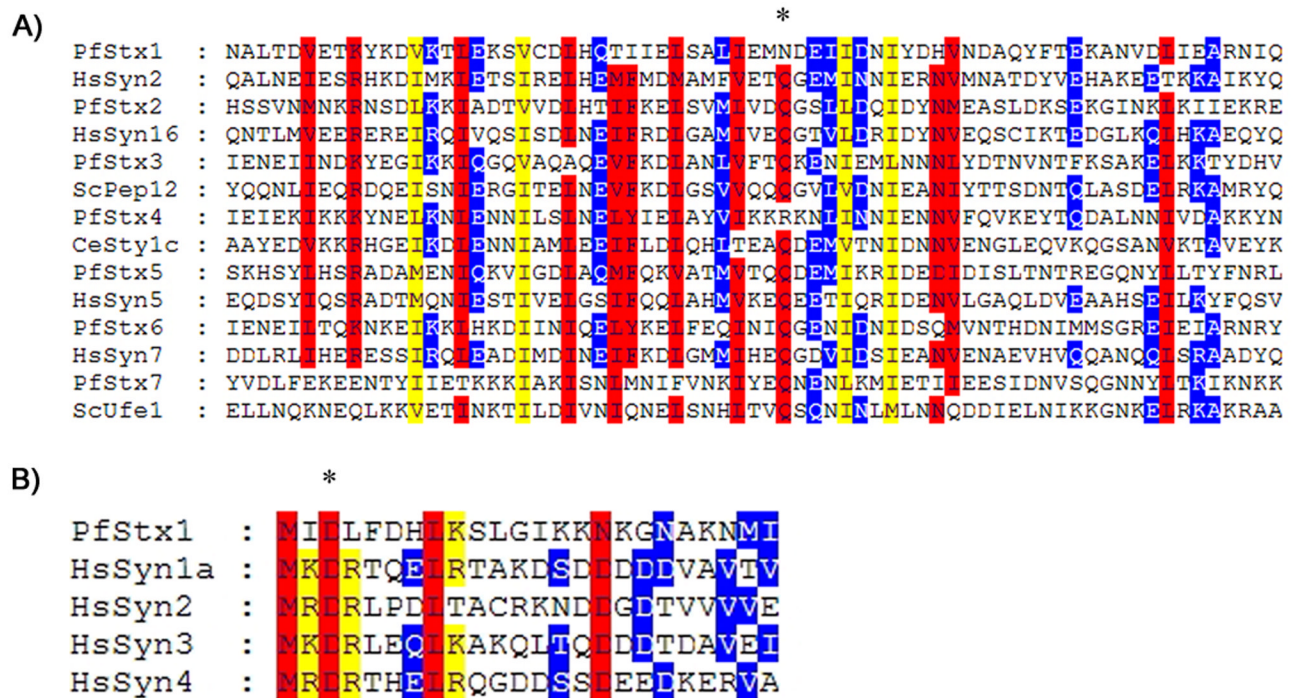
Figure 1. Sequence alignment of PfStx proteins with other eukaryotic Qa-SNAREs
A) Sequence alignment of the SNARE domain of all seven *P. falciparum* Qa-SNAREs and their closest Qa-SNARE homolog from other eukaryotes. Red indicates regions where there is 80% or more similarity, yellow indicates regions where there is 60% or more similarity, and blue indicates regions where there is 40% or more similarity. The conserved Q amino acid at the core of the SNARE domain is indicated by an asterisk. B) Alignment of the N-terminus of PfStx1 with the conserved N-terminal sequence of HsSyn1a, HsSyn2, HsSyn3, HsSyn4, all of which are plasma membrane Qa-SNAREs. The first 10 amino acids of HsSyn1a interact with the SM family protein Munc18 [18], and the D3 amino acid residue (asterisk) is essential for this interaction. Residues critical for the HsSyn1a-Munc18 interaction, including D3, are conserved in PfStx1.
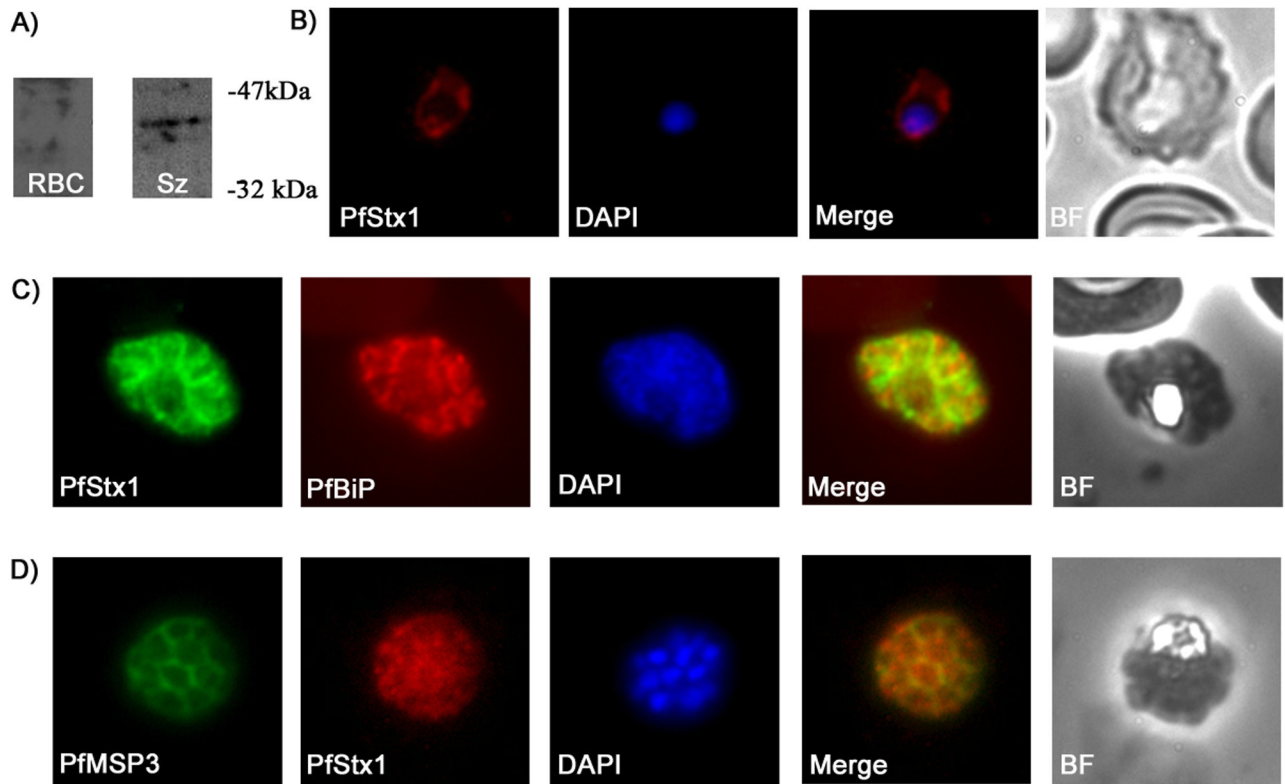
Figure 2. PfStx1 is localized proximal to the *P. falciparum* plasma membrane
A) Western blot of *P. falciparum* protein extracts run on a 12% SDS gel and probed with anti-PfStx1 antiserum. Polyclonal anti-PfStx1 antiserum was raised against a his-tagged fragment of PfStx1. A partial sequence of *PfStx1* (forward primer 5'-CGGGATCCGAATTAAGTATAGAATTAAAT-3', reverse primer 5'-CGGGAATTCTTATCTAGCTTCTATTAAATCTAC-3') was cloned into expression plasmid pRSETA (Invitrogen) and transformed into *Escherichia coli* BL21-DE3 pLysS. The resulting protein was expressed as a hexa-his fusion protein and was purified from *E. coli* pellets by resuspending the pellets in binding buffer (8M urea, 50mM TRIS pH 8.2, 100mM NaCl and 10mM imidazole) and passing through a Hi-Trap chelating HP column (Amersham Biosciences). Columns were washed 5 times with binding buffer and purified protein was eluted with increasing amounts of imidazole ranging from 30mM to 500mM. The purified protein was then used to immunize rabbits and rats (Cocalico Biological, Inc.). Lanes: RBC – total SDS extract of uninfected erythrocytes; Sz - total SDS extract from *P. falciparum* 3D7 schizont stage parasites. B–D) Immunofluorescence microscopy of fixed smears of *P. falciparum* parasites using anti-PfStx1 antiserum. Fixed air-dried smears of *P. falciparum* 3D7 were fixed in a 1:1 acetone methanol solution at −20°C for 30 minutes, washed with 1X PBS and then probed with the primary antisera. Secondary antisera (Molecular Probes) labeled with a fluorophore were used for visualization on an Olympus BX60 fluorescence microscope, and images were merged with Adobe Photoshop 5.0. In trophozoite stage parasites PfStx1 (red) has a plasma membrane like staining pattern and does not appear to be exported into the erythrocyte (B). In schizont stage parasites, PfSyn1 (green) again has a peripheral location that is distinct from the ER marker PfBiP (in red) (C). PfStx1 (red) colocalizes with PfMSP3 (in green), a plasma membrane marker in schizont stage parasites (D), confirming its predicted location proximal to the plasma membrane.

Table 1

Plasmodium falciparum Qa-SNAREs

Seven *P. falciparum* Qa-SNAREs were identified by searching the *P. falciparum* genome (www.plasmodb.org) using either t-BLASTn or Psi-BLAST programs using both the full length sequences and the more conserved C-terminal SNARE domain of Qa-SNAREs from the genomes of *S. cerevisiae, D. melanogaster*, *C. elegans*, and *H. sapiens*. *P. falciparum* Qa-SNAREs are referred to as Stx in keeping with the gene name of the original Qa-SNARE, syntaxin 1 (HsStx1). The accession numbers and closest known Qa-SNARE homologs for each PfStx gene are listed, as are the *P. falciparum* SNARE gene names previously assigned in a non-Qa specific manner [16]. A possible intracellular location of each *P. falciparum* Qa-SNARE is predicted based on their closest eukaryotic Qa-SNARE homologue.

P. falciparum Qa-SNAREs	*P. falciparum* SNAREs [16]	Closest Qa-SNARE homolog	Predicted intracellular location
PfStx1 (PFB0480w)	PfSyn17	HsSyn2, DmSyn1a	Plasma membrane
PfStx2 (PFL2070w)	PfSyn16	HsSyn16, DmSyn16	Trans-Golgi
PfStx3 (PFL0505c)	PfSyn2	ScPep12, HsSyn7	Endosome
PfStx4 (Pf14_0300)	PfSyn11	CeSyn1c, CeSyn13	Plasma membrane?
PfStx5 (MAL13P1.169)	PfSyn5	HsSyn5, DmSyn5	Cis-Golgi
PfStx6 (PF11_0052)	PfSyn13	HsSyn7, ScSso1	Food Vacuole, Plasma membrane?
PfStx7 (MAL13P1.365)	PfSyn18	ScUfe1	ER