A comparative analysis of trypanosomatid SNARE proteins

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The Kinetoplastida are flagellated protozoa evolutionary distant and divergent from yeast and humans. Kinetoplastida include trypanosomatids, and a number of important pathogens. Trypanosoma brucei, Trypanosoma cruzi and Leishmania spp. inflict significant morbidity and mortality on humans and livestock as the etiological agents of human African trypanosomiasis, Chagas’ disease and leishmaniasis respectively. For all of these organisms, intracellular trafficking is vital for maintenance of the host–pathogen interface, modulation/evasion of host immune system responses and nutrient uptake. Soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs) are critical components of the intracellular trafficking machinery in eukaryotes, mediating membrane fusion and contributing to organelle specificity. We asked how the SNARE complement evolved across the trypanosomatids. An in silico search of the predicted proteomes of T. b. brucei and T. cruzi was used to identify candidate SNARE sequences. Phylogenetic analysis, including comparisons with yeast and human SNAREs, allowed assignment of trypanosomatid SNAREs to the Q or R subclass, as well as identification of several SNARE orthologues with those of opisthokonts. Only limited variation in number and identity of SNAREs was found, with Leishmania major having 27 and T. brucei 26, suggesting a stable SNARE complement post-speciation. Expression analysis of T. brucei SNAREs revealed significant differential expression between mammalian and insect infective forms, especially within R and Qb-SNARE subclasses, suggesting possible roles in adaptation to different environments. For trypanosome SNAREs with clear orthologs in opisthokonts, the subcellular localization of TbVAMP7C is endosomal while both TbSyn5 and TbSyn16B are at the Golgi complex, which suggests conservation of localization and possibly also function. Despite highly distinct life styles, the complement of trypanosomatid SNAREs is quite stable between the three pathogenic lineages, suggesting establishment in the last common ancestor of trypanosomes and Leishmania. Developmental changes to SNARE mRNA levels between blood stage and procyclic life stages suggest that trypanosomes modulate SNARE functions via expression. Finally, the locations of some conserved SNAREs have been retained across the eukaryotic lineage.

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1. Introduction

Kinetoplastids are flagellated protozoa of the Excavata supergroup and evolutionarily distant from model eukaryotes such as fungi, animals and plants [1]; the order contains many pathogenic species. Major kinetoplastid pathogens include the African trypanosomes, represented by Trypanosoma brucei, causing African trypanosomiasis in humans and nagana in livestock and largely restricted to sub-Saharan Africa, the American trypanosome, Trypanosoma cruzi, the etiological agent of Chagas’ disease, and also the Leishmania species, that cause various forms of leishmaniasis in Southern Europe, Africa, Asia and America [2]. Globally, approximately 25 million people are affected by trypanosomatid infections, while the number at risk exceeds 250 million [3]. Available kinetoplastid genome sequences indicate significant conservation of gene complement and synteny [4], but different lineages cause highly distinct diseases and survive in discrete biological environments; for example T. brucei is exclusively extracellular while T. cruzi and Leishmania major invade host cells [5].

Intracellular trafficking is responsible for the transport and sorting of lipid and protein cargo between membrane-bound intracellular compartments. Trafficking requires spatially and temporally co-ordinated
protein–protein interactions and is fundamental to cell growth and differentiation, nutrient uptake, immune evasion, signaling and many other processes [6]. In trypanosomes, intracellular trafficking is especially important in evading the mammalian host immune system and maintaining the surface proteome. Specifically the copy numbers of proteins and other molecules that participate directly in immune defense or other pathogenesis associated events are significantly varied during life cycle progression. A potent example of this phenomenon is *T. brucei*, where antigenic variation [7] requires high-level surface expression of the variant surface glycoprotein, but in addition, immune evasion is augmented by recycling of surface antigens and immunoglobulin degradation via the endocytotic pathway [8,9].

Among the key proteins mediating intracellular trafficking are the Rab and ARF small GTPases, vesicle coat proteins and soluble N-ethylmaleimide-sensitive factor attachment protein receptors or SNAREs [10]. SNAREs are 10–30 kDa, subcellular compartment-specific, type II membrane proteins, characterized by a highly conserved SNARE motif, a ~70 amino acid block comprising hydrophobic heptad repeats [11,12]. The SNARE motif, usually located towards the C-terminus and connected to a trans-membrane domain by a short linker, is critical for forming the SNARE complex during membrane fusion [13]. Many SNARE proteins also contain additional domains at the N-terminus, that serve to regulate SNARE complex assembly, and some SNAREs deviate from this prototypical organization. For example, *Homo sapiens* SNAP-23, SNAP-25, SNAP-29, Syn11 and Saccharomyces cerevisiae Ykt6 all lack a trans-membrane domain but are membrane anchored via prenylation or palmitoylation [14,15]. Human SNAP-25, which contains two SNARE motifs, attaches to membranes by non-covalent association with trans-membrane domain SNAREs [16,17].

Classification of SNAREs is based on the conservation of an amino acid residue in the central polar layer of the coiled-coil SNARE complex [18]. This residue is either a glutamine (Q) or an arginine (R), and defines Q- and R-SNARE subclasses [19]. Based on the relative positions of these critical residues within the SNARE complex, Q-SNAREs are further sub-classified into Qa-, Qb-, Qc-, Qd- and Qe-SNAREs [11]. Qc-SNAREs are also differentiated by their N-terminal organization. Syntaxins and a few Qb- and Qc-SNAREs contain an Habc domain three-helix bundle [20] that is thought to act as a binding site for regulatory SM proteins [19]. The Habc domain may also fold back onto the SNARE domain to give a ‘closed’ conformation, preventing interaction of cognate SNARE partners [21]. R-SNAREs are sub-classified into short vesicle-associated membrane proteins (VAMPs; brevins) and long VAMPs (longins) based on the presence of a short and variable domain at the N-terminus respectively [22].

Comparative genomic and phylogenetic analyses have, to some degree, defined a SNARE complement for the last eukaryotic common ancestor (LECA) and thus set expectations for the complement likely present in a given eukaryotic genome. Five Qa-SNARE subfamilies appear to be ancient [54]: Syntaxin 5, 16, 18, as well as the SynPM and SynEl clades, which have undergone lineage-specific expansions in animals and yeast [55,56]. The LECA Qb-SNARE complement consists of at least Vti1, Gos1, Bos1 and Sec20, while the Qc complement holds Syntaxin 6, 8, and Bet 1 as a minimum [57]. Finally, the R-SNARE complement consists of three longin subfamilies Sec22, Ykt6 and Vamp7. Vamp7 is expanded in several eukaryote lineages [55,58], and also gave rise to the brevins, Vamp1-6, 8 and Snc1/2, which are believed to be opisthokont-specific [59].

Given that intracellular membrane transport is so critical for immune evasion and other cellular processes in trypanosomes, a detailed understanding of the process is clearly of importance. The roles of many proteins in trafficking in *T. brucei* and additional trypanosomatids have been described [23,24], but the contributions made by members of the SNARE repertoire remain to be elucidated. Building on an earlier investigation of *L. major* SNAREs [25], we identified and classified the putative SNARE complement in predicted proteomes of *T. brucei* and *T. cruzi*. These, together with L. major and opisthokont reference sequences, allow a classification for trypanosome SNAREs to be derived. Additionally, we predicted the domain structures and investigate the expression profile of the *T. brucei* SNAREs. Finally, by determining the subcellular location of a select cohort of the SNAREs that are conserved between trypanosomes, animals and fungi, we provide evidence for retention of a similar location of orthologous SNAREs across the eukaryota.

### 2. Materials and methods

#### 2.1. Genome searches for candidate SNARE open reading frames

The predicted proteomes of *T. brucei* and *T. cruzi* were obtained from EuPathDB (http://eupathdb.org/eupathdb/) and formatted into BLAST searchable databases. Validated *Leishmania major* SNAREs [25] were used to query the formatted databases using BLASTP [26] with cut-off E-value of 0.0001, given the short length of the proteins. Domain content predictions for the retrieved sequences were generated at the Pfam [27] and PROSITE [28] domain databases. Only sequences predicted to contain the SNARE domain were retained as potential homologues. These sequences were aligned using MUSCLE [62] and manually edited using Jalview [63] and subsequently used to create a Hidden Markov Model (HMM) profile that was used to exhaustively reinterrogate the *T. brucei* and *T. cruzi* genomes for distant homologues using the HMMER package [29]. Additionally, in cases where one kinetoplastid ortholog of a clade was not initially identified, BLASTp searches using the relevant sequences of the other trypanosomatids were performed. Trans-membrane (TM) domain topology prediction was performed using SMART [60]. Fold recognition was performed using the fold threading software PHYRE (www.sbg.bio.ic.ac.uk/–phyre).

#### 2.2. Sequence alignment and phylogenetic reconstruction

Multiple sequence alignments were generated using MUSCLE [30] and manually edited in MacClade v4.08 to only retain unambiguously aligned regions [31]. Phylogenetic reconstruction was performed using two separate methods. To obtain the best Bayesian trees, topology and posterior probability values, the program MrBayes v3.2.1 [32] was used with the following run parameters; prestat aamodelpr = fixed(WAG); mmncngen = 10,000,000; samplefreq = 1000; nchains = 4; startingtree = random; sumpburnin = 2500; sumburnin = 2500. Posterior probabilities were used as a measure of node robustness. All calculations were checked for convergence by running the analysis several times, with results compared using test div. The model of sequence evolution prior to each PhyML analysis was determined using Prot-Test v3.2.1 [34] and included corrections for rate variation used to determine the best substitution model and invariable sites where applicable. Trees were rendered using FigTree v1.2 [35]. To identify SNAREs that are conserved between trypanosomes, humans and yeast, opisthokont landmark sequences were included in the analyses. In some cases selected opisthokont-specific duplications of subfamilies were excluded to alleviate phylogenetic artifact. For R-SNAREs, only longin landmark sequences were used.

#### 2.3. Trypanosome cell culture

Bloodstream form cells of *T. brucei* Lister 427 (wild-type 427, WT427) and the derived single marker bloodstream (SMB) line [36] were cultured in HMI-9 complete medium (Gibco) [37] supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Biosera), 100 U/mL penicillin, 100 U/mL streptomycin (Gibco) and 2 mM l-glutamine (Gibco), maintained at 37 °C with 5% CO2 in a humid atmosphere in non-adherent culture flasks with vented caps. Cells were maintained in non-adherent culture flasks with vented caps. Cells were maintained...
at densities between $10^5$ and $5 \times 10^6$ cells/mL. Ectopic expression of plasmid constructs was maintained using G418 antibiotic selection at 2.5 μg/mL [38].

2.4. Recombinant DNA constructs

Putative trypanosome SNAREs Tb927.9.3820 (TbQc1B), Tb10.70.7410 (TbVAMP7C), Tb927.10.14200 (TbSyn5) and Tb09.211.3920 (TbSyn16B) were PCR amplified from trypanosome 427 genomic DNA using Vent DNA polymerase (New England BioLabs). For hemagglutinin (HA)-tag fusion constructs of Tb927.9.3820 (TbQc1B), and Tb10.70.7410 (TbVAMP7C), the PCR products were cloned into the Bsf expression vector pXS5, containing sequence for a C-terminal HA-epitope, using HindIII and Apal or HindIII and Clal using the following primers: Tb927.9.3820-F5′-GCAAGCTTATGCGATCTGAAAGG and Tb927.9.3820-R3′-CCGG GCCCCCCAGACTATGTGTATCCG; Tb10.70.7410-F5′-GGAAGCATTATGC AGGGGAGCAACAAA and Tb10.70.7410-R3′-GGGGGCCCCCTTCCTTCTTCT TTTTT. For hemagglutinin (HA)-tag fusion constructs of Tb927...
endosomally-associated SynE [42] and plasma membrane localized SynPM [41,43]. Other kinetoplastid Qa-SNAREs fell into well-supported clades, but these lack clear opisthokont members. Additionally, we observed an L. major–specific duplication of the SynPM Qa SNARES (LmSynPM1 and LmSynPM2). In the Qb-SNARE tree, only the GOSR1 clade resolved with robust support as containing both kinetoplastid and opisthokont sequences. Other tritryp SNAREs in this subclass form well-defined 1:1:1 orthologous relationships, but
without identifiable opistokont affiliation. In the Qc-SNARE tree (Fig. 1C), a clade uniting the opistokont Bet1 sequences with two robustly supported kinetoplastid subclades was reconstructed, although without internal resolution. Additionally, we observed three Qc clades (Qc-1-3) for which opistokont orthologs could not be robustly assigned. Qc1 and Qc2 were also reconstructed as encompassing two separate subclades each containing the three trypanosomatids examined. In the R-SNARE tree (Fig. 1D), three opistokont SNAREs formed clades with trypanosomatid sequences; ER-Golgi Sec22, involved in anterograde transport from the ER, the Golgi-vacuole localized Ykt6, and endosomal Vamp7. Additionally, the clade of R1 contained proteins from all three trypanosomatids, but was not robustly assignable to an opistokont ortholog (data not shown).

From these reconstructions we observed a few cases of genome-specific expansion and also of failure to identify a particular ortholog. However, overall we largely found a 1:1:1 ortholog among the trypanosomatid SNAREs, indicating general stability of the SNARE complement. This contrasts with the Rab GTPases which are represented by a larger cohort in T. cruzi and L. major than in T. brucei. In just under 50% of the cases, we were unable to identify an opistokont ortholog for a particular clade of kinetoplastid SNAREs. Whether this is due to true biological novelty or failure of the phylogenetic methodology to resolve relationships between distantly related proteins awaits more in depth analysis, possibly with improved phylogenetic methods when they become available. Nonetheless, we were able to identify ortholog relationships of trypanosomatid SNAREs with opistokont sequences in 10 of 19 cases; these trypanosome SNAREs are candidates for assuming equivalent cellular functions.

3.2. T. brucei SNARE architecture

The majority of T. brucei, T. cruzi and L. major SNAREs exhibit prototypic SNARE features, i.e. a C-terminal trans-membrane domain linked to a SNARE motif by a short linker, plus, in several, a helical N-terminal domain (Fig. 2). However, several SNAREs in both T. brucei and T. cruzi do not conform to this standard architecture. One of the non-prototypic T. brucei candidates, Tb927.8.3470 (TbQb2), is predicted to contain two putative SNARE domains at the N- and C-termini respectively. This is a unique finding given that such an architecture of N- and C-terminal SNARE domains has been reported for SNAP-23, SNAP-25, SNAP-29, Sec9p and Spo20p, but these are mainly restricted to animals, higher plants, fungi, and ciliates [61]. Further investigation of this...
T. brucei SNARE is warranted given that the L. major homologue (LmjF.23.1740 (LmQb2)) appears to only contain the N-terminal domain [25].

Several T. brucei SNAREs, Tb927.9.14080 (TbYKT6), Tb927.11.15400 (TbR1) and Tb927.10.11980 (TbSyn16A), lack a C-terminal trans-membrane domain, necessitating an alternate mechanism for membrane association, for example by acylation [44]. CSS-Palm [45] and PrePS [46] algorithms predict C-terminal palmitoylation sites for TbYKT6 (Cys 201 and 202) and Tb927.11.16320 (TbQa1) (Cys 282). The T. cruzi and L. major orthologs of TbYKT6 are also predicted to be palmitoylated, at Cys201 and Cys202 respectively, while the Tb927.11.16320 (TbQa1) orthologs (TcCLB.506211.230 (TcQa1) and LmjF.19.0120 (LmQa1)) are predicted to be palmitoylated at Cys294 and 272 respectively. TbR1 is also predicted to be palmitoylated at a central residue (Cys996). In addition to acylation, SNAREs lacking a trans-membrane domain may insert into membranes via hydrophobic interactions with proteins possessing a trans-membrane motif as has been reported for SNAP-25 [16].

All T. brucei Qa-SNAREs were predicted to contain the N-terminal Habc domain (Fig. 2). This domain regulates SNARE activity by preventing coiled-coil formation. Although generally restricted to the Qa-SNAREs, the Habc domain was putatively identified in several Qc-SNAREs (TbQc1A, TbQc2A and TbQc3). Finally, the R-SNAREs appeared to possess the canonical domain structure for this subclass. Only in TbR1 did we fail to predict a longin domain.

3.3. Differential expression of T. brucei SNAREs

To investigate if the identified T. brucei SNARE genes are transcribed, real-time PCR was performed, using gene-specific primers, against total RNA from both the bloodstream (BSF) and procyclic forms (PCF) of the parasite. Significant levels of transcription were found for the entire cohort. While our transcriptome data suggests that TbSyn5, TbR1 TbSyn16A and TbQb2A are constitutively expressed, a subset of T. brucei SNAREs are differentially expressed at the mRNA level between lifecycle stages. Further, consistent with earlier data [47], we also find that the SNAREs analyzed in this study are differentially expressed, with the majority being up-regulated in the BSF relative to the PCF (Fig. 3). This dynamic expression is also consistent with the earlier study by Bestiero et al. [25], which demonstrated that L. major SNAREs are differentially regulated, suggesting that this may be a general phenomenon of the trypanosomatid SNARE cohort. As membrane trafficking requirements are variable between life stages, these transcriptional changes may reflect significant changes to individual transport steps. In T. brucei, SNAREs must play a critical role in recycling of VSG, a process that requires both high rates of endocytosis as well as recycling/exocytosis. While we did observe strong up-regulation of TbVAMP7B, we saw little evidence for changes in the expression of the remaining cohort of putative endosome-associated SNAREs. By contrast to the endosomal SNAREs, there is prominent up-regulation in the BSF of TbSec22 and TbYKT6 which suggests potential modulation of specific ER exit pathways, and which may be coupled to the presence of two Rab1 orthologs and a Rab 2 ortholog in T. brucei and hence complexity in ER exit [48].

3.4. Subcellular localization of trypanosome SNAREs

The sequences of several differentially expressed T. brucei SNAREs that were also found to have an ortholog in either H. sapiens or S. cerevisiae. TbSyn16B, TbVAMP7C, TbQc2A, TbVAMP7A, TbSyn5 and TbQc1B were chosen for genomic tagging in order to identify the subcellular location of the protein [47]. Multiple attempts to fuse a C-terminal hemagglutinin (HA) epitope tag to TbVAMP7A and TbQc2A were unsuccessful, but the remaining four SNAREs were successfully tagged and expressed. Intracellular localization of the HA-tagged SNARE proteins was assessed by staining with an anti-HA antibody and by co-staining cells using a selection of markers, including early endosomal epsinR, the lysosome marker p67, the plasma membrane and endosomal markers ISG65 and ISG75 and the endosomal/post-Golgi proteins clathrin, Rab5 and Golgi-located GRASP [49–53].

Immunofluorescence revealed juxtaposition between TbVAMP7C and ISG65, clathrin, epsinR and Rab5A, with the majority of the immunoactivity localized to the region between the nucleus and kinetoplast (Fig. 4). These co-localizations indicate a possible endosomal localization for TbVAMP7C, consistent with the phylogenetic analysis. TbQc1B demonstrated a location very close to the posterior face of the nucleus, while expression levels were rather low and as a consequence localization was equivocal (Fig. 5). TbSyn5 is juxtaposed to GRASP (Fig. 6), suggesting localization to Golgi-associated structures. This was expected given the orthologous relationship with the Golgi located human Syn5 (Fig. 1A). Additionally, LmSyn5 has been experimentally localized at the Golgi [25], while TbSyn16B is also juxtaposed to the Golgi (Fig. 6). This was expected given the orthologous relationship with the Golgi localized human STX16 (Syn16) (Fig. 1A). These data suggest that for these three SNAREs where orthologous relationship could be established, the locations of the trypanosome proteins suggest retention of targeting specificity with their mammalian and yeast orthologs.

Fig. 3. Steady state mRNA levels of T. brucei SNAREs. Triplicate RNA samples from wild type BSF and PCF cells were subjected to qRT-PCR. BSF and PCF expression levels are represented by red and blue bars respectively. Data normalization for RNA was relative to β-tubulin and telomerase reverse transcriptase (TERT) proteins. Note error bars are absent from the PCF data set as this is set at 1.0 and variance was less than 5% throughout.
4. Conclusions

The SNARE repertoire appears well conserved between \textit{L. major}, \textit{T. brucei} and \textit{T. cruzi}, with a restricted number of losses or expansions between these organisms. It is therefore unlikely that the SNARE complement plays a major role in defining the highly divergent life styles and specific pathogenesis and immune evasion mechanisms of these parasites. This contrasts with a more restricted Rab protein repertoire in African trypanosomes compared with \textit{T. cruzi} and \textit{Leishmania}, and further underscores the importance of Rab proteins in mediating evolution of new trafficking pathways. Any contribution from SNAREs to adaptation of the trypanosomatid trafficking system is likely in expression levels, specific amino acid changes and/or precise mechanistic aspects. Endocytosis is significantly developmentally regulated in African trypanosomes, but significantly we observed little up-regulation of SNAREs assigned as endocytosis orthologs. Experimental investigation of the three SNAREs conserved between trypanosomatids and opisthokonts suggests that the subcellular locations of the orthologs are conserved. This mirrors the conservation observed among the vast majority of Rab GTPases, and while location and function need not been fully concordant, this evidence does suggest a likely functional equivalence has been retained, in at least some aspects; direct experimental evidence is needed to verify this hypothesis. Further our phylogenetic evidence indicates that a substantial proportion of trypanosome SNAREs may be orthologous with those in other eukaryotes and consequently possibly perform similar functions. SNAREs could therefore serve as excellent cellular markers in many organisms for the definition of intracellular compartments.

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