Journal of Cell Science 112, 3091-3101 (1999)
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JCS0440

A novel protein targeting domain directs proteins to the anterior cytoplasmic face of the flagellar pocket in African trypanosomes

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Accepted 26 June; published on WWW 26 August 1999

SUMMARY

The flagellar pocket of African trypanosomes is a critical sorting station for protein and membrane trafficking, and is considered to be an Achilles' heel of this deadly pathogen. Although several proteins, including receptors for host-derived growth factors, are targeted specifically to the flagellar pocket, the signals responsible for this restricted subcellular localization are entirely unknown. Using T lymphocyte triggering factor-green fluorescent protein (TLTF[1]-GFP) fusion proteins, we demonstrate that an internal 144 amino acid domain of TLTF from *Trypanosoma brucei* is sufficient for directing GFP to the cytoplasmic side of the anterior flagellar pocket. Immuno-gold electron microscopy reveals that the TLTF-GFP fusion protein is located in an electron dense structure that immediately abuts the anterior flagellar pocket membrane. The amino acid sequence of the TLTF targeting domain does not resemble previously characterized protein trafficking signals, and random mutagenesis reveals that flagellar pocket targeting is conferred by a structural motif, rather than a short, contiguous array of amino acids. The aberrant sorting of two mutant proteins into the flagellum, and the targeting of a related human protein to the plus end of the trypanosome's cytoskeletal microtubules, lead us to suggest that flagellar pocket targeting involves interactions with the trypanosome cytoskeleton. The finding that TLTF-GFP is restricted to the anterior, cytoplasmic face of the flagellar pocket membrane, suggests that there is structural heterogeneity in the membrane of this organelle.

Key words: Trypanosome, Protein targeting, GFP, Flagellar pocket

INTRODUCTION

African trypanosomes are uni-flagellated protozoan parasites that cause African sleeping sickness, a fatal disease with devastating health and economic consequences. These parasites are indigenous to sub-Saharan Africa, where it is estimated that 300,000 people are infected annually and 55 million are at risk (WHO, 1996). In the last three decades there has been a dramatic and steady rise in the incidence of this disease, and in some provinces within Angola and Democratic Republic of Congo the number of deaths due to sleeping sickness now equals or exceeds those due to AIDS (WHO, 1996). In addition to the tremendous human health burden posed by these deadly pathogens, their rampant infection of wild and domestic livestock makes it nearly impossible to sustain a healthy economy in endemic regions.

Due to the demands imposed by their parasitic lifestyle, trypanosomes exhibit a highly organized surface membrane, which serves as the interface between parasite and host. This surface membrane can be divided into three structurally and functionally distinct domains: the flagellar membrane, the plasma membrane, and the flagellar pocket (Balber, 1990). Although these domains constitute a contiguous lipid bilayer, they each have their own characteristic properties, which stem from their specialized functions (Balber, 1990). The parasite's single flagellum is encased by its own membrane and is attached to the cell body via regularly spaced junctional complexes of unknown composition (Seebeck et al., 1990; Vickerman and Preston, 1976). The plasma membrane (pellicle) surrounds the cell and is subtended by a corset of closely spaced, interconnected microtubules that make up the cell cytoskeleton (Seebeck et al., 1990; Vickerman and Preston, 1976). These subpellicular microtubules extend the entire length of the cell, being absent only beneath the membrane of the flagellar pocket and in a small region at the extreme posterior end of the cell, where they converge in a ring-like structure (Hemphill et al., 1991; Seebeck et al., 1990). The flagellar pocket, is a dynamic, membrane-enclosed organelle formed by an invagination of the plasma membrane at the point where the the flagellum emerges from the cell body (Overath et al., 1997; Webster and Russell, 1993).

The trypanosome's unique cellular architecture has important consequences for the cell biology of the organism and its interactions with the host. In particular, the longitudinal array of subpellicular microtubules acts as a barrier to membrane scission and fusion events, thereby confining

Table 1. Quantitation of phenotypes exhibited by targeting domain mutants*

	Control		Strong defect				Moderate defect					
	WT	GFP	V169A	N132D	K191I	K191N E196G	K249E	1208T	D125G E198G	K231N	I216V	E158V
% Targeted	**73.3**	0.0	23.8	11.9	3.3	5.4	32.5	26.3	**61.6**	**54.5**	**52.2**	**47.3**
% Green	14.1	**100.0**	**68.8**	**62.8**	**60.0**	7.3	**45.3**	**42.4**	15.3	28.5	20.4	28.1
% Grn/Trg	11.1	0.0	0.5	20.0	5.0	0.5	21.1	9.5	6.9	15.6	12.7	6.2
% Flagellum	0.4	0.0	0.0	4.4	**29.7**	**86.8**	0.0	18.0	12.0	1.0	12.1	9.6
% Speckled	1.1	0.0	6.9	0.9	2.0	0.0	1.2	3.8	4.2	0.3	2.7	8.9
(*n*)	(923)	(330)	(202)	(320)	(300)	(205)	(342)	(316)	(216)	(288)	(339)	(146)

*The ten mutant proteins that exhibited the most prominent defects in the primary mutant screen were independently transfected into trypanosomes at least three times and the number of cells with a given phenotype were scored. Data from all transfections of each mutant were totalled, and the percentage of each phenotype was calculated by dividing the total number of cells expressing a phenotype by the total number of transfected cells counted. The amino acid substitution(s) in each mutant is indicated at the top of the corresponding column, and the predominant phenotype is shown in bold. The total number of cells scored (*n*) for each mutant is given in the last row of the Table. Results of parallel analyses from transfections with the wild-type TLTF targeting domain fused to GFP (WT) and with GFP alone (GFP) are also shown. Phenotypes are shown in Fig. 5a ('Targeted'), 5d ('Green'), 5b and c ('Grn/Trg') 5e and f ('Flagellum'). A small percentage of cells in each transfection exhibited numerous spots of GFP ('Speckled' in Table 1; not shown in Fig. 5).

endocytosis and secretion to the specialized membrane of the flagellar pocket (Overath et al., 1997; Webster and Russell, 1993). In essence, the flagellar pocket is an extracellular compartment that is secluded from the host environment by close apposition of the flagellar and plasma membranes in desmosome-like adhesion zones (Balber, 1990; Vickerman and Preston, 1976). By restricting host-parasite exchange of macromolecules to the flagellar pocket, the parasite minimizes exposure of critical surface proteins. Indeed, the finding that trypanosome-encoded receptors for host-derived growth factors, e.g. tranferrin and low density lipoprotein, are located at the flagellar pocket has led to the idea that this organelle may represent an Achilles' heel of the parasite (Coppens et al., 1988; Salmon et al., 1994; Webster and Grab, 1988).

The protein and membrane sorting demands imposed on the flagellar pocket are immense. However, despite the critical importance of this organelle in facilitating macromolecular traffic, as well as the identification of several proteins that are targeted to the flagellar pocket (Coppens et al., 1988; Lee et al., 1990; Salmon et al., 1994; Vaidya et al., 1997; Webster and Grab, 1988), no one has yet identified any signals in these proteins that mediate flagellar pocket targeting. TLTF is a trypanosome protein that was identified by its ability to stimulate host T cells (Olsson et al., 1993). We recently cloned the cDNA for TLTF from *Trypanosoma brucei* and found that the protein was located primarily at or near the flagellar pocket (Vaidya et al., 1997). Although the precise physiological role of TLTF is unclear, we have taken advantage of its subcellular distribution to identify amino acid sequences responsible for flagellar pocket targeting.

MATERIALS AND METHODS

Trypanosome strains, culture conditions and DNA transfections

Procyclic trypanosomes (clone YTAT1.1, obtained from E. Ullu, Yale University, New Haven, CT) were cultured in Cunningham's SM medium (Cunningham, 1977), supplemented with 20% heat-inactivated fetal calf serum. DNA transfections were performed as described previously (Vaidya et al., 1997) with the exception that the electroporation medium contained 120 mM KCl_2, 0.15 mM $CaCl_2$, 9.2 mM K_2HPO_4, 25 mM Hepes, 2 mM EDTA, 4.75 mM $MgCl_2$, 69 mM sucrose, pH 7.6. Transfected cells were transferred to fresh culture medium and examined for GFP fluorescence 16-28 hours after transfection. Quantitative studies of GFP-expressing cells (Table 1) were conducted using an Olympus BHT fluorescent microscope, equipped with a BH-RFL 100 watt mercury lamp. Fluorescent and transmitted images were captured using a BioRad MRC-1024 Laser Scanning Confocal Head mounted on a Nikon Optiphot microscope with a 60× oil-immersion objective (1.4/NA). Transmitted and fluorescent images were captured simultaneously on separate channels using BioRad Laser Sharp software, then merged using Adobe PhotoShop (Adobe Systems, Inc., San Jose, CA).

Stably transfected trypanosomes expressing the HI-GFP fusion protein (see Fig. 4) were generated by transfection of YTAT 1.1 procyclic trypanosomes with 25 μg of plasmid pHD496:HIGFP (see below) linearized at the unique *Not*I site. Transfected cells were allowed to recover for 24 hours and then supplemented with 30 μg/ml hygromycin. Hygromycin resistant cells grew out after about 3 weeks of drug selection.

Plasmid construction

All molecular biology procedures were conducted using standard methods (Sambrook et al., 1989). The sequence of all cloned DNA fragments and chimeric genes were confirmed by automated fluorescent DNA sequencing at the DNA sequencing core facility at the University of Iowa. The *T. brucei* expression vectors pHD:GFPm3 and pHD:GFPm3-TLTF have been described previously (Vaidya et al., 1997). To generate the GFP-AB and GFP-CD fusion constructs, the full-length TLTF gene was excised from pHD:GFPm3-TLTF as an *Eco*RI-*Bam*HI fragment, and replaced with TLTF fragment AB (codons 1-257) or CD (codons 196-453). The AB and CD DNA fragments were generated by PCR-amplification of the corresponding TLTF codons, using oligonucleotide primers to introduce *Eco*RI (5′) and *Bam*HI (3′) sites at the ends of each DNA fragment. For fragment AB, the *Bam*HI primer contained a stop codon immediately following TLTF codon 257. To fuse TLTF fragments to the N terminus of GFP, the desired region of the TLTF open reading frame was PCR-amplified using appropriate primers to introduce *Hin*dIII sites at each end, as well as an ATG start codon at the 5′ end. These fragments were cloned into the *Hin*dIII site of pHD:GFPm3 to yield in-frame GFP fusions. The plasmid pHD:HX-GFPm3 was created to allow for the directional cloning of TLTF targeting domain mutants (vide infra) as *Hin*dIII/*Xba*I fragments. To generate pHD:HX-GFPm3, the GFPm3 open reading frame (Cormack et al., 1996) was PCR-amplified with GFP-specific primers containing contiguous *Hin*dIII and *Xba*I restriction sites immediately preceding the GFP start codon, and a

*Bam*HI site following the GFP stop codon. This DNA fragment was cloned into the *Hin*dIII/*Bam*HI sites of pHD1 (Hug et al., 1993) to generate pHD:HX-GFP.

To generate plasmid pHD496:HI-GFP, the chloramphenicol acetyl transferase (CAT) gene was excised from pHD496 (Biebinger et al., 1996) as a *Hin*dIII/*Bam*HI fragment and replaced with the wild-type HI-GFP chimeric construct (see above). pHD496 utilizes a rRNA promoter to drive expression of the reporter and hygromycin resistance genes and includes sequences from the T. brucei ribosomal RNA spacer region harboring unique *Not*I and *Eco*RV sites (Biebinger et al., 1996). Linearization with *Not*I or *Eco*RV allows for homologous integration into the rRNA spacer region and selection with hygromycin yields cells stably expressing the reporter gene.

Protein sequence alignments and analyses were performed using the University of Wisconsin GCG Software package (Devereux et al., 1984). Protein secondary structure predictions were made using the Chou-Fasman (Chou et al., 1974) and Robson (Garnier et al., 1978) indeces, and the MacStripe COILS program (version 2.0b1; MITDK matrix), which employs the Lupas algorithm for predicting coiled-coil domains (Lupas et al., 1991).

Immunoelectron microscopy

For immunoelectron microscopy, cells were fixed in 200 mM PIPES, 0.5 mM $MgCl_2$, pH 7.0 with 4% paraformaldehyde for 45 minutes at 4°C. The cells were washed, pelleted in 10% gelatin in PIPES buffer, and infiltrated in 20% polyvinylpyrrolidone, 1.86 M sucrose in PIPES buffer. The pellet was plunge frozen in liquid nitrogen and sections were cut on an MT7/CR21 cryomicrotome. Sections were placed on grids and blocked in 5% goat serum, 5% fetal calf serum in PIPES buffer (block buffer) and incubated with rabbit anti-GFP (Clontech) diluted 1:500 in block buffer. The grids were washed in block buffer and incubated with secondary antibody: 18 nm gold-conjugated goat anti-rabbit IgG (Jackson Immunoresearch Labs). Grids were washed sequentially with block buffer, PIPES buffer, and H_2O prior to embedding in 0.3% uranyl acetate in 20% methyl cellulose.

PCR-mediated mutagenesis of the TLTF targeting domain

Random mutations were introduced into DNA encoding the TLTF flagellar pocket targeting domain, fragment ‘HI’ (Fig. 2; codons 114-257), by PCR-mediated mutagenesis (Leung et al., 1989). For mutagenic PCR, TLTF codons 114-257 were PCR-amplified with primers ‘TLTF-H’ (5′-CCCAAGCTTATGGATGAAAGTCACCGT-CTG-3′) and ‘TLTF-I-Xba’ (5′-GCTCTAGACTGCTTCATCTGCG-CTATTTC-3′). Primer TLTF-H contains a *Hin*dIII restriction site and an ATG start codon in front of codon 114 of TLTF. Primer TLTF-I-Xba contains an *Xba*I restriction site following codon 257 of TLTF. Mn^{2+} was added to the PCR reaction in 0.1 mM increments (from 0 to 2 mM final concentration), and the Mg^{2+} concentration was adjusted accordingly to maintain a total concentration of [Mn^{2+} + Mg^{2+} = 2 mM]. PCR efficiency was significantly reduced in preliminary experiments at 0.4-0.5 mM Mn^{++}, indicating that the fidelity of Taq polymerase would be duly compromised (Leung et al., 1989). To assess the number of mutations introduced into the TLTF targeting domain, we digested the PCR products with *Hin*dIII and *Xba*I, shotgun cloned the digested fragments into the *Hin*dIII/*Xba*I sites of pHD:HX-GFP and sequenced 20 representative clones. The 0.4 and 0.5 mM Mn^{2+} clones contained an average of one to two nucleotide substitutions each, and we chose these pools to serve as the source of mutants for screening.

Targeting domain mutant screen

Plasmids containing mutagenized TLTF sequences were isolated according to the Qiagen protocol (Qiagen, Chatsworth, CA) and transfected into procyclic trypanosomes (Vaidya et al., 1997). Transfection efficiencies ranged from 20% to 40% for any given transfection. Transfected cells were examined by fluorescence microscopy 16-28 hours after transfection and the pattern of GFP localization was compared to that seen in cells transfected with plasmids encoding fusions between GFP and the wild-type TLTF targeting domain or with plasmids encoding GFP alone. The level of GFP expressed in transiently transfected trypanosomes remained relatively constant over the 16-28 hour time frame.

Cloning of the human ‘targeting domain’ into pHD-HX-GFP

A human EST clone with sequence similarity to TLTF (Vaidya et al., 1997) was obtained from Research Genetics (Huntsville, AB). The sequence of the relevant region (i.e., that corresponding to codons 114-257 of TLTF; Fig. 7a) was determined and used as a template for PCR amplification to introduce *Hin*dIII and *Xba*I sites at the 5′ and 3′ ends, respectively. This ‘HumanHI’ DNA fragment was cloned into the *Hin*dIII/*Xba*I sites of pHD:HX-GFP to generate pHD:HumanHI-GFP.

RESULTS

Subcellular targeting of TLTF is mediated by an internal protein targeting signal

We previously showed that a GFP-TLTF fusion protein is primarily located in punctate structures that are concentrated in the flagellar pocket region of the cell (Vaidya et al., 1997). Unlike varient surface protein (VSG) and procyclic acidic repeat protein (PARP), which transit through the flagellar pocket en route to the plasma membrane, TLTF does not have a hydrophobic N-terminal signal sequence (Vaidya et al., 1997) that might allow it to get to the flagellar pocket via the classical secretory pathway. To identify TLTF amino acids that mediate its subcellular localization, we constructed a systematic series of TLTF open reading frame deletions, fused the partial TLTF-encoding fragments to the GFP gene and examined the subcellular location of the resultant chimeric proteins in transiently transfected trypanosomes. We first divided the TLTF gene into two overlapping fragments of approximately equal size, one encoding the first 257 amino acids of TLTF (fragment AB) and one encoding the C-terminal 258 amino acids (fragment CD). The localization of the GFP-AB fusion protein is similar to that of the full length fusion protein (Fig. 1b,d), whereas the GFP-CD fusion protein remains in the cytoplasm (Fig. 1c), as does GFP alone (Fig. 1a). Hence, the first 257 amino acids of TLTF contain all the information necessary for flagellar pocket targeting.

The results discussed above were obtained using GFP-TLTF chimeras in which TLTF amino acids were fused to the C terminus of GFP. After discovering that the targeting signal is located within the N-terminal portion of TLTF, we generated new constructs which placed either the entire TLTF protein, or its first 257 amino acids in front of GFP. These studies indicated that the ability of TLTF (or its N-terminal 257 amino acids) to direct GFP to the flagellar pocket is independent of whether TLTF is placed in front of or behind GFP (data not shown).

To further delimit the targeting signal, we conducted a deletion analysis of fragment AB (Fig. 2a). GFP fusion proteins containing the first 53 amino acids (fragment EF in Fig. 2a; not shown in Fig. 2b) or the first 113 amino acids (fragment EY) of TLTF are evenly distributed throughout the cytoplasm (Fig. 2), indicating that TLTF does not contain a cryptic signal sequence at its N terminus. On the other hand, when TLTF amino acids 114-257 (fragment HI) are fused to

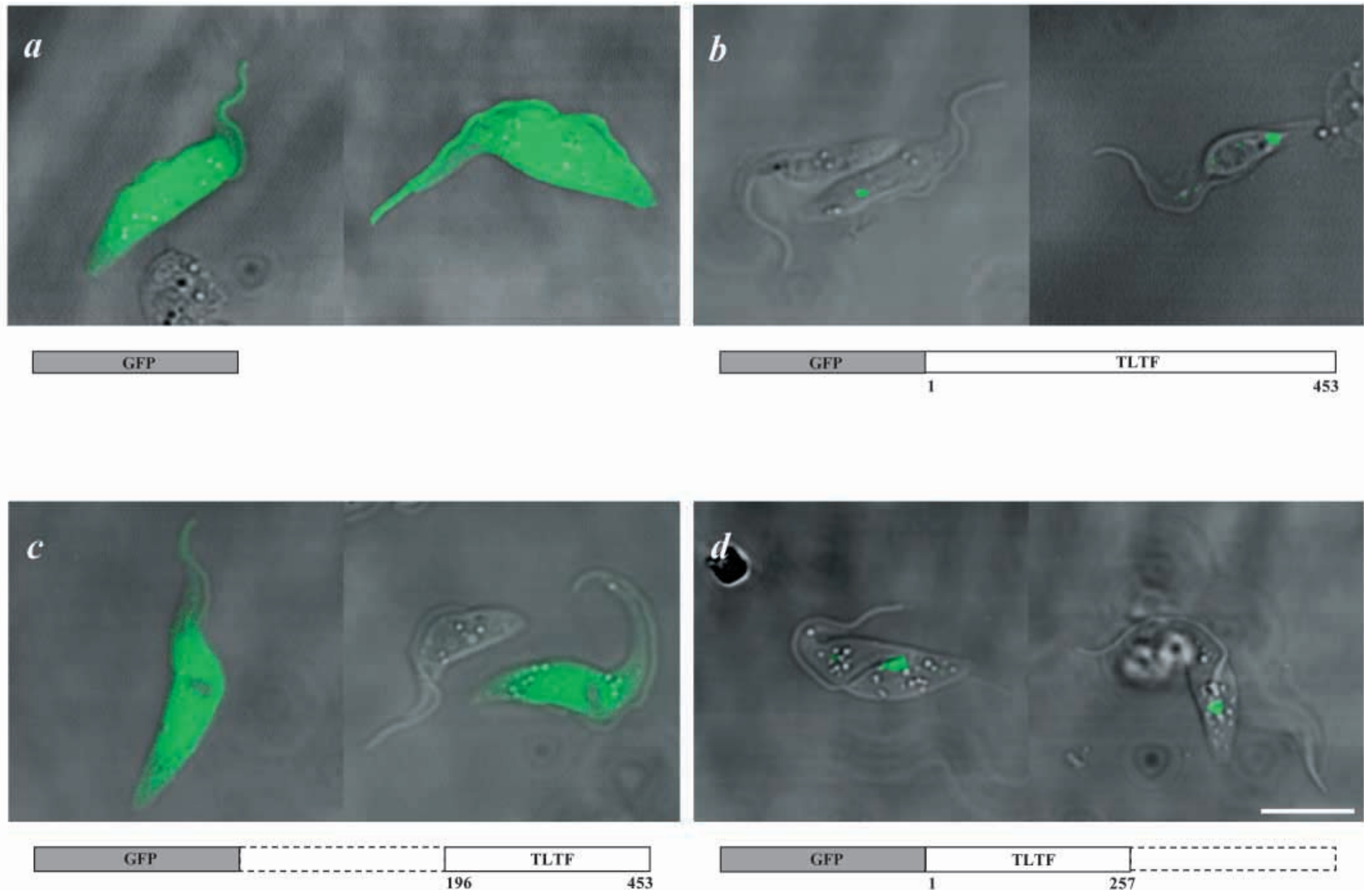


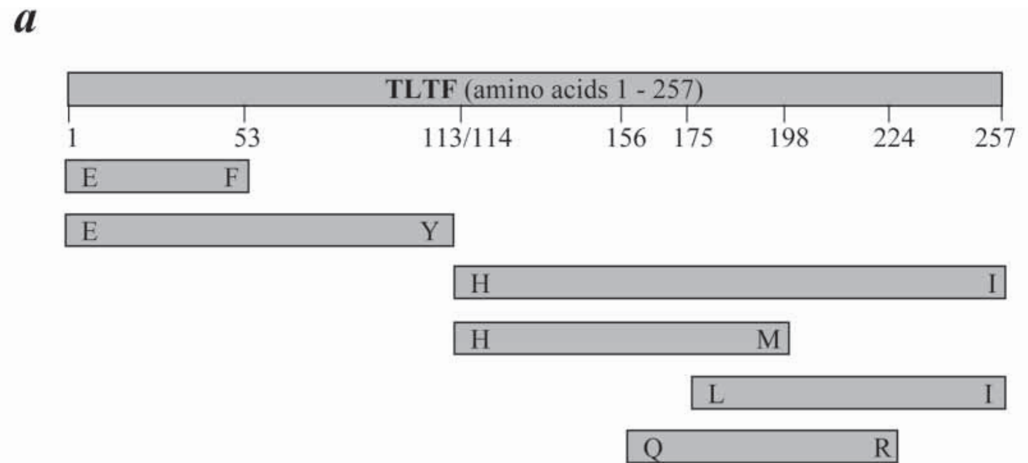
Fig. 1. The N-terminal 257 amino acids of TLTF are sufficient for targeting to the flagellar pocket region of African trypanosomes. Procyclic trypanosomes expressing the fusion proteins indicated below each panel were examined by fluorescence microscopy. Two representative examples of GFP-expressing cells are shown in each case. GFP alone is distributed evenly throughout the cytoplasm (a). GFP fused to full-length TLTF (amino acids 1-453) is localized to the flagellar pocket region (b). GFP fused to the C-terminal portion of TLTF (amino acids 196-453) is distributed throughout the cytoplasm (c). GFP fused to the N-terminal portion of TLTF (amino acids 1-257) is localized to the flagellar pocket region (d). Fusion proteins are drawn schematically below each panel with gray bars representing GFP and white bars representing TLTF. Dashed lines indicate portions of TLTF that are absent from the chimeric proteins. In b and c two cells that have not taken up plasmid DNA are also visible. Fluorescent and transmitted images were captured simultaneously using laser scanning confocal microscopy and then superimposed. The white scale bar corresponds to 10 µm.

GFP, the protein is localized to the flagellar pocket region (Fig. 2).

Further deletion analysis indicated that residues 114-257 constitute a minimal targeting domain, since removing amino acids from either end of this fragment resulted in cytoplasmic localization (Fig. 2). To rule out the possibility that the TLTF portion of these chimeric proteins was proteolytically removed after translation, whole-cell protein extracts from transfected cells were subjected to western blot analysis (Fig. 3). In all cases, a fusion protein of the expected size is produced, regardless of whether or not the protein is targeted to the flagellar pocket. Western blot analysis of other proteins used in this study (Figs 1 and 2) likewise revealed that the correct size protein was expressed (not shown). Therefore, failure to localize to the flagellar pocket is not due to proteolytic removal of the TLTF portion of these chimeric proteins.

Ultrastructural localization of the TLTF(HI)-GFP fusion protein

The location of the HI-GFP fusion protein was exmined at the ultrastructural level by immunoelectron microscopic analysis of trypanosomes stably transfected with the minimal TLTF targeting domain (fragment HI in Fig. 2) fused to GFP. Almost all of the anti-GFP antibody label was associated with a dense oval structure that abuts the flagellar pocket membrane (Fig. 4). Irrespective of the angle of section this structure appears to be surrounded by a limiting membrane (although this is not always well defined), and has no obvious internal structure beyond its dense matrix. The GFP-containing structure is always observed as a single structure and, when it is possible to determine the orientation of the cell, is invariably found on the anterior, cytoplasmic face of the flagellar pocket membrane. The ultrastructural location of the anti-GFP gold particles is entirely consistent with fluorescence microscopy results (e.g. Fig. 2). A low level of gold-labelled antibody occurs in the cytosol around the flagellar pocket (Fig. 4A) and in the mitochondrion (not shown), but no intense labelling comparable to that near the flagellar pocket was observed in any preparations. Cell sections that did not include the flagellar pocket region very rarely contained gold-labelled antibody.



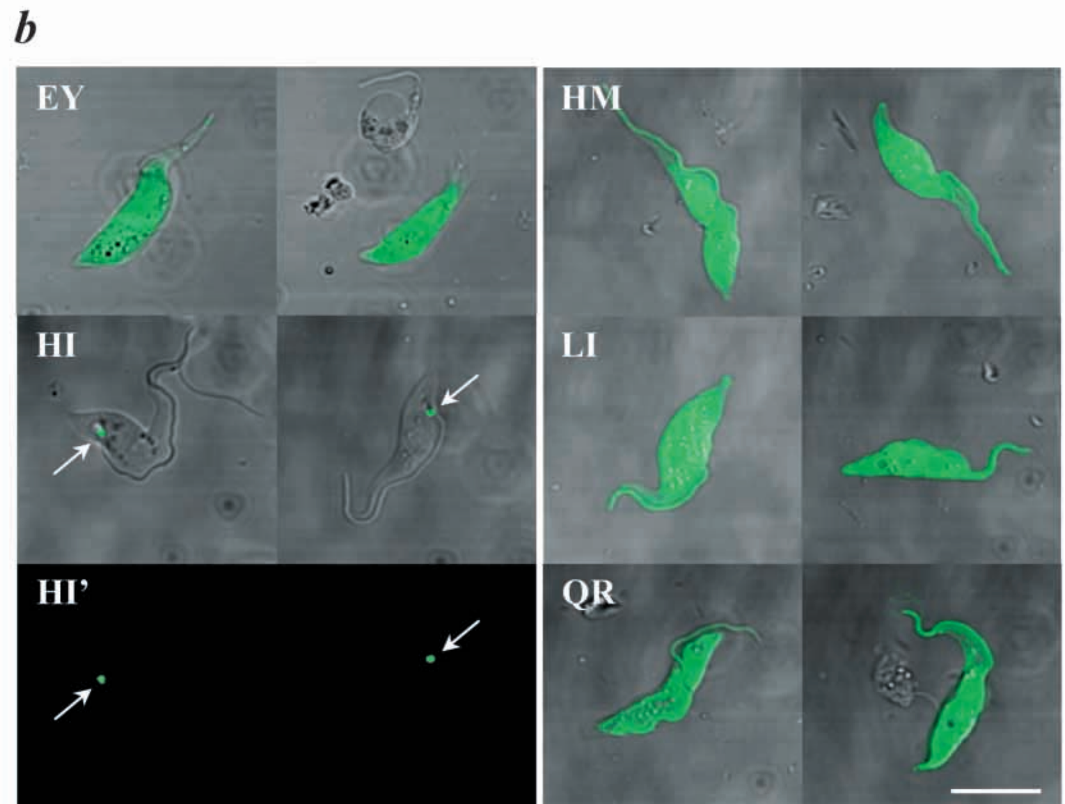


Fig. 2. TLTF amino acids 114-257 constitute a minimal targeting domain. (a) A schematic diagram of the N-terminal 257 amino acids of TLTF (fragment AB in Fig. 1) together with the TLTF polypeptide fragments that were fused to the N terminus of GFP. The number of the first and last amino acid of each TLTF polypeptide fragment is noted. (b) Live trypanosomes expressing the indicated TLTF polypeptide fragments fused to GFP. Fragment HI is the only polypeptide that directs GFP to the flagellar pocket (arrows). Fluorescent and transmitted images were captured simultaneously using laser scanning confocal microscopy and then superimposed. For HI, the same two cells are shown as a merged (HI) or fluorescence only (HI′) image. GFP fusion proteins were expressed in transiently transfected trypanosomes as described in the Methods section. The white scale bar corresponds to 10 µm.

Control preparations of wild-type trypanosomes indicated that background labelling was below 9%, and tended to be confined to the nucleus (not shown).

Identification and characterization of targeting domain mutants

To determine whether flagellar pocket localization is mediated by a short, contiguous stretch of amino acids within the targeting domain, or whether it requires recognition of a particular three-dimensional structure of the entire domain, we examined the effect of random point mutations in the TLTF targeting domain. Our aim was to determine whether targeting defects could be linked to changes within a small stretch of amino acids, or result from mutations that are scattered throughout the entire targeting signal.

To generate targeting domain mutants, we employed PCR-mediated mutagenesis (Leung et al., 1989). This method exploits the fact that the fidelity of DNA polymerase is compromised when Mn^{2+} is substituted for Mg^{2+} as a cofactor during the polymerization reaction. We selected a Mn^{2+} concentration that yielded an average of one to two nucleotide substitutions in each PCR product. Individual PCR products were cloned and sequenced to identify mutations, and transfected into trypanosomes to assess targeting competence.

In the first round of screening, we transfected each of 26

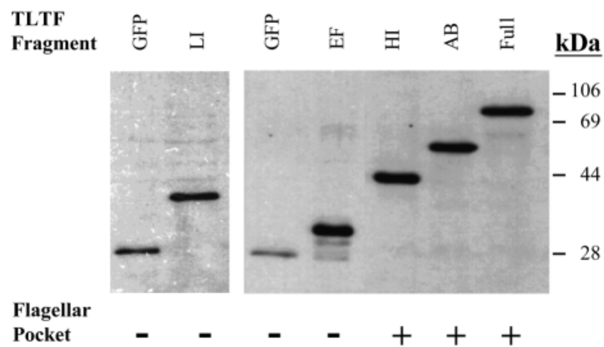


Fig. 3. TLTF-GFP fusion proteins are not abnormally degraded. Protein extracts from whole cells of transiently transfected trypanosomes were resolved by SDS-PAGE and analyzed by western blot analysis using polyclonal anti-GFP antibodies (Clontech, Palo Alto, CA). The TLTF fragment that is fused to GFP (see Fig. 2) is indicated at the top of each lane. 'Full' refers to the full-length TLTF-GFP chimera, and 'GFP' refers to GFP alone. The position of molecular weight standards is indicated on the right, and flagellar pocket (+) or cytoplasmic (−) localization is indicated at the bottom of the gel. All fusion proteins migrate at their expected sizes, indicating that the TLTF portions of the proteins have not been proteolytically removed.

mutant DNA constructs into wild-type trypanosomes and examined 35 to 152 transfected cells per transfection. 3 mutant DNA constructs were found to have stop codons in the TLTF coding region. Trypanosomes transfected with these mutants displayed the expected phenotype, i.e., no GFP fluorescence (not shown). An additional 2 mutants had more than three missense mutations each, and were not characterized further. The remaining 21 mutants fell into one of three categories based on the observed GFP targeting phenotype: (i) predominantly wild-type targeting (>70% of transfected cells show flagellar pocket localization of GFP); (ii) moderately defective targeting (30-70% wild-type localization); and (iii) strongly defective targeting (<30% wild-type localization). We selected ten mutants with the most prominent defects for additional characterization.

Phenotypes exhibited by trypanosomes expressing targeting domain mutants are shown in Fig. 5. These include trypanosomes in which mutant proteins retain wild-type targeting (Fig. 5a); those that target inefficiently, sending some GFP to the flagellar pocket, yet retaining a significant amount of GFP in the cytoplasm (Fig. 5b,c); those which are green throughout the cell (Fig. 5d); and finally, those with a striking phenotype in which GFP is sent into the flagellum itself (Fig. 5e,f). In this class of mutants it appears that the mutant proteins can still get to the vicinity of the flagellar pocket, i.e. near the opening of the flagellum, but have lost the ability to recognize an important interaction that specifies retention there (see Discussion).

To quantitate targeting defects, each mutant clone was individually introduced into wild-type trypanosomes by 3 to 4 independent transfections, and a total of 146 to 342 transfected cells were examined for each mutant (Table 1). The distribution of phenotypes observed with the wild-type targeting domain fused to GFP (wt), as well as with GFP alone (GFP), are shown for comparison. The mutants can be grouped into general categories based on the relative penetrance of their targeting defects. Four mutants (V169A, N132D, K191I, and K191N/E196G) show a strong defect, whereas the other six mutants exhibit a more moderate defect. The moderately defective group can be further subdivided into mutants whose predominant phenotype is cytoplasmic GFP (K249E, I208T), and those in which the predominant phenotype remains flagellar pocket-directed GFP.

It is noteworthy that the two mutants which show a strong tendency to misdirect GFP into the flagellum (K191I and K191N/E196G) share an amino acid substitution at lysine 191. The simplest interpretation of these results is that amino acids

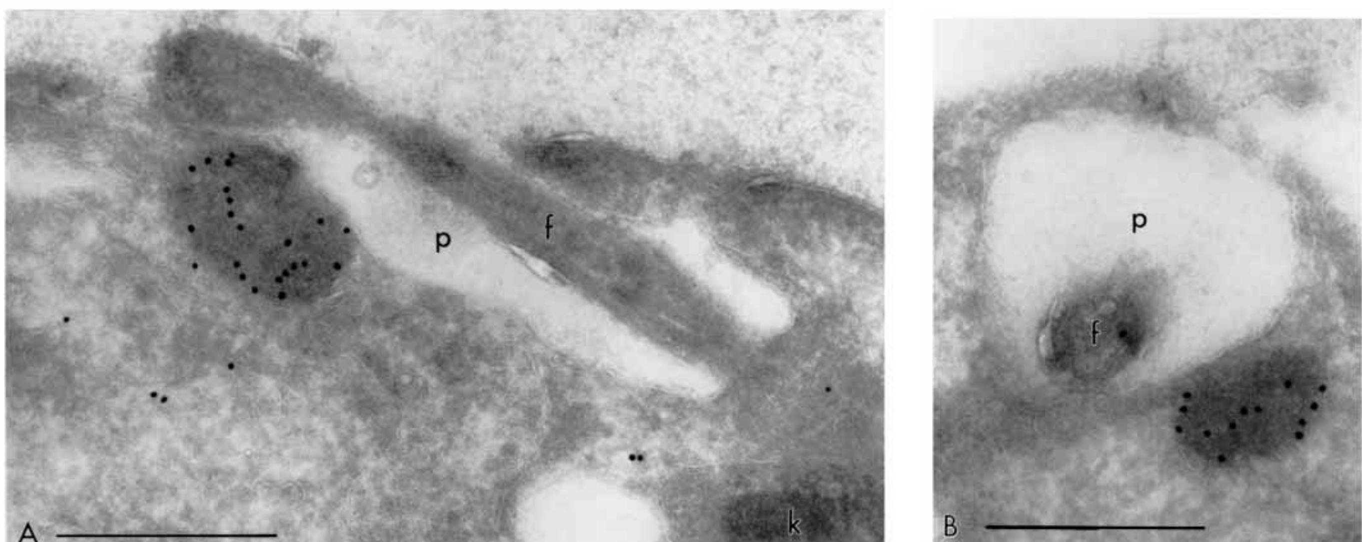


Fig. 4. Immunoelectron micrographs of *T. brucei* cells stably expressing the HI-GFP fusion protein. A and B show cryosections of cells expressing the HI-GFP fusion protein (Fig. 2) after probing with rabbit anti-GFP followed by 18 nm gold-conjugated, goat anti-rabbit IgG. The vast majority of label is associated with a dense, ovoid structure that abuts the flagellar pocket (p). This dense body appears to have a limiting membrane and is always on the anterior side of the flagellar pocket. (k, kinetoplast; f, flagellum). Scale bars, 0.5 μm.

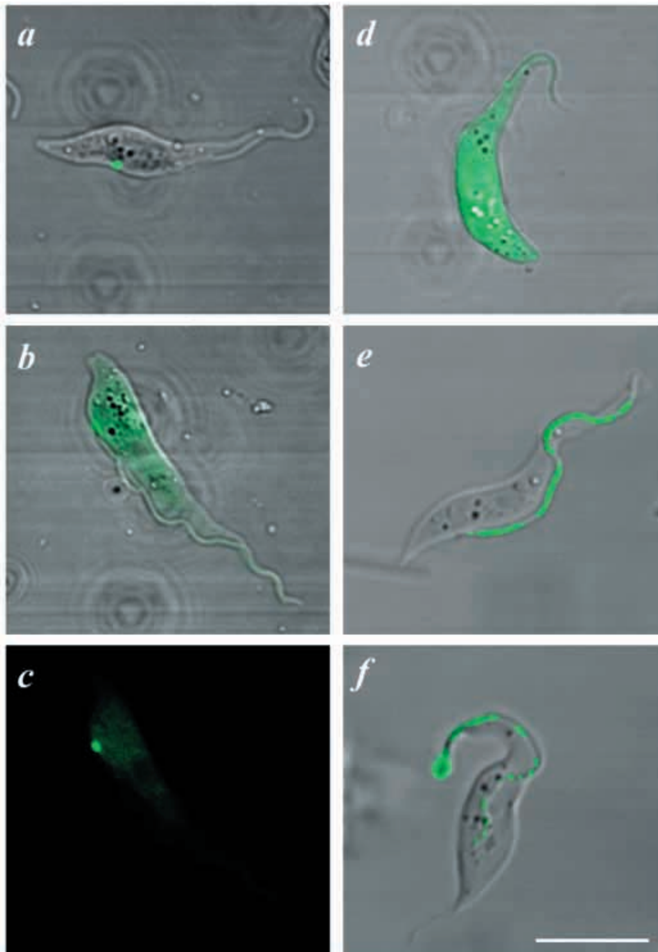


Fig. 5. Representative phenotypes of flagellar pocket targeting domain mutants. Representative examples of the phenotypes exhibited by TLTF targeting domain mutants are shown. (a) Wild-type targeting; (b,c) inefficient targeting, as indicated by the intense GFP fluorescence near the flagellar pocket with diffuse fluorescence in the cytoplasm; (d) loss of targeting; and (e,f) flagellum targeting. c shows the fluorescent image of the cell shown in b. The bar corresponds to 10 μm.

191-196 are part of a signal which specifies retention at the anterior cytoplasmic face of the flagellar pocket, and that the protein is aberrantly delivered into the flagellum if this information is comprimised.

Flagellar pocket targeting involves recognition of a conformational signal

Fig. 6 shows the positions and identities of amino acid substitutions in ten TLTF targeting domain mutants having aberrant GFP localization, along with several amino acid substitutions that do not affect targeting (data not shown). Both types of mutations are dispersed throughout the entire 144 residue targeting domain, suggesting that flagellar pocket localization involves recognition of a conformational signal that must be presented in its proper context to be effective. Analysis of the Chou/Fasman (Chou et al., 1974) and Robson indices (Garnier et al., 1978) for predicted protein secondary structure, indicates that the TLTF targeting domain has a high alpha-helical content (Fig. 7a), consistent with the possibility that a three-dimensional structural motif is the basis for recognition. The targeting domain is also predicted to have a high probability of forming a coiled coil structure based on the COILS prediction algorithm of Lupas (Lupas et al., 1991). Interestingly, of the deletion mutants examined (Fig. 2), only the HI fragment retains the integrity of two predicted coiled coil domains in this region of the protein. In the absence of more specific structural information, it is likely premature to try to interpret the effect of each mutation on a point-by-point basis.

A human 'TLTF'-like protein targets GFP to punctate structures at the plus end of the pelicular microtubules

We previously identified in the dbEST sequence database a potential human protein with high amino acid sequence similarity (60% similarity, 34% identity) to TLTF (Vaidya et al., 1997). Many of the conserved amino acids occur in discreet segments of these two proteins. In particular, a large portion of the flagellar pocket targeting domain is nearly identical to the corresponding region of the human protein (Fig. 7a; Vaidya et al., 1997). In this region 84% of 68 contiguous residues are either identical or are conservative substitutions. Furthermore, eight of the ten targeting mutants identified in our screen have amino acid substitutions at conserved residues. Finally, although the primary structure of the N-terminal portion of the TLTF targeting domain is not conserved between the human and trypanosome protein, the predicted secondary structure is conserved (Fig. 7a). These observations led us to ask whether the human counterpart to the trypanosome targeting domain could function in trypanosomes. Indeed, we find that GFP fused to a portion of the human protein analogous to the TLTF flagellar pocket

Fig. 6. TLTF targeting mutations are distributed throughout the entire targeting domain. The minimal TLTF targeting domain (amino acids 114-257) is represented as a bar. Regions conserved in a human protein (see Fig. 7a) are shaded gray. Non-conserved regions are black. Mutations that alter targeting are shown above the bar. Mutations that do not affect targeting are shown below the bar. Multiple amino acid substitutions that are present in a single mutant are either drawn together or are marked with the same number of asterisks (e.g. D125G and E198G occur in the same mutant). Both types of mutations (those that affect targeting and those that do not) are distributed throughout the entire targeting domain.

Fig. 7. Targeting phenotype of a human TLTF homologue. A putative human TLTF homologue was identified on the basis of amino acid sequence similarity. (a) Amino acid alignment of the trypanosome TLTF targeting domain with the corresponding region of a putative human homologue. Similar amino acids are boxed and positions of identity are shaded gray. The latter half of the targeting domain represents one of the most conserved regions in these two proteins. Helices beneath the amino acid alignment represent regions of the proteins predicted to be alpha-helical by the Chou-Fasman and Robson algorithms for predicting protein secondary structure (Chou et al., 1974; Garnier et al., 1978). Black underlines represent regions in the trypanosome protein with a high probability of forming coiled-coils (Lupas et al., 1991). (b) To determine whether targeting information is conserved between the trypanosome and human protein, the region of the human protein corresponding to the trypanosome targeting domain was cloned in front of GFP, and the corresponding fusion protein was expressed in trypanosomes as described in the Methods section. Transfected trypanosomes were visualized as described in Fig. 1. Three representative transfected cells are shown in b. The bar corresponds to 10 μm.

targeting domain, exhibits a discrete subcellular distribution that is similar but not identical to TLTF-GFP (Fig. 7b). Like TLTF-GFP, the human-GFP fusion protein is localized to punctate structures. In this case however, the protein is restricted to the extreme posterior of the cell, rather than to the flagellar pocket. Interestingly, the extreme posterior end of the cell and the flagellar pocket share the conspicuous feature that they are the only domains of the trypanosome surface membrane which do not have an underpinning of pellicular microtubules (Hemphill et al., 1991; Seebeck et al., 1990). Hence, the human and trypanosome proteins share sequences that target them to architecturally analogous subcellular locations, although the human protein lacks the information for specific routing to the flagellar pocket. This result suggests that the TLTF targeting signal contains multiple layers of information, and that each of these layers must be correctly interpreted for the protein to arrive at the flagellar pocket. It also suggests that some components of the TLTF targeting machinery may be conserved in mammalian cells. Indeed, we find that both the trypanosome and human targeting domains confer similar patterns of punctate localization in mammalian cells (K. L. Hill and J. E. Donelson, unpublished observation).

DISCUSSION

We have identified a 144 amino acid domain that specifies protein targeting to the anterior cytoplasmic face of the flagellar pocket in African trypanosomes. The amino acid sequence of this domain does not resemble any previously characterized protein targeting signals, and is located in the middle of the protein from which it is derived. A screen for random point mutations revealed that amino acid substitutions that disrupt targeting, as well as those that do not, are dispersed throughout the entire 144 amino acid domain. This result indicates that targeting is not mediated by a linear array of a few amino acids, but requires a particular conformational signal that must be presented in its proper context in order to function correctly. Consistent with this idea, the targeting domain contains two regions predicted to form coiled coils (Fig. 7a), which are common motifs for mediating protein-

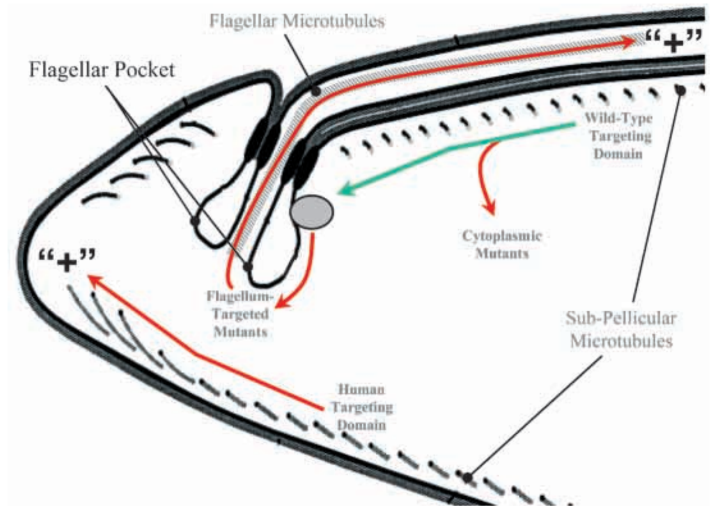


Fig. 8. Schematic diagram of the trypanosome's posterior end, showing a proposed model for flagellar pocket targeting. In this model, TLTF interactions with trypanosome microtubules (direct or indirect) contribute to flagellar pocket targeting (green arrow). A conformational signal in the wild-type targeting domain recognizes a particular feature of the flagellar pocket, retaining the protein specifically at the anterior side of the flagellar pocket's cytoplasmic face (gray oval). Point mutations in the targeting domain lead to aberrant targeting (red arrows). Some mutants loose all targeting capacity (cytoplasmic mutants) while the flagellum-targeted mutants and the human protein interact with microtubules, but cannot recognize the flagellar pocket 'retention signal' and thus continue along microtubule tracks, in the plus end direction ('+'). (See text for further discussion.) Trypanosome schematic adapted from Bangs (1998).

protein interactions (Lupas, 1991). Of the deletion mutants examined, only the minimal targeting domain (HI in Fig. 2) retains the integrity of both these predicted coiled coil domains. Since our mutagenesis procedure was not saturating, we cannot rule out the possibility that there may be small contiguous stretches of amino acids that are especially critical. However, given the relatively even distribution of mutations that disrupt targeting, it would appear that a proper three-dimensional structure of the entire targeting domain is essential. This is the first report of a protein domain that specifically directs protein targeting to the trypanosome's flagellar pocket region. In *T. brucei,* the transferrin receptor, LDL receptor, and CRAM (cysteine rich acidic membrane protein) are all localized to the flagellar pocket by unknown mechanisms (Coppens et al., 1988; Lee et al., 1990; Salmon et al., 1994; Webster and Grab, 1988). We compared the amino acid sequence of TLTF to those available for the transferrin receptor and CRAM, but did not find any significant sequence similarities (not shown).

Many protein sorting decisions in eukaryotic cells are based upon recognition of short amino acid sequences. Examples include the tetrapeptide signal specifying endoplasmic reticulum retention (Bangs et al., 1996; Pelham, 1991), a tripeptide signal for peroxisome (Gould et al., 1989) and glycosome (Sommer et al., 1992) import, and tyrosine-based motifs for cell surface receptor internalization (Ohno et al., 1995). Our results show that the flagellar pocket localization signal from TLTF is fundamentally different from these short protein targeting signals. Rather, it is more akin to the N-terminal signal sequence of classically secreted proteins (von Heijne, 1982, 1983), the internal structural signal that routes interleukin-1β into the non-classical secretory pathway (Siders and Mizel, 1995), or the bipartite N-terminal signal that directs cytoplasm to vacuole trafficking of aminopeptidase I in *S. cerevisiae* (Oda et al., 1996). These signals have in common the fact that they impart targeting information *via* a structural motif, rather than a particular conserved amino acid sequence.

Immunoelectron microscopic examination of cells stably expressing GFP fused to the minimal TLTF targeting domain revealed that the protein is found almost exclusively in an electron dense, membrane-enclosed structure that abuts the flagellar pocket membrane, on its cytoplasmic side (Fig. 4). When it is possible to determine cell orientation, the GFP-containing structure is always located at the anterior side of the flagellar pocket. Although this electron-dense structure is unlike previously characterized vesicles in trypanosomes, it is possible that overexpression of the fusion protein leads to an enlargement of an endogenous structure that is not easily observed in wild-type cells. An enlargement of endogenous membrane vesicles has been reported in trypanosomes overexpressing a *T. brucei* Rab2 protein (Field et al., 1998). We are currently working to express the fusion protein using *T. brucei* expression vectors that allow for controlling the expression level of reporter genes (Wirtz et al., 1999).

Thus far, our efforts to detect TLTF by immunofluorescence and immunoelectron microscopy using antibodies that recognize this protein in western blots have been unsuccessful. Hence we have employed GFP, which has proved to be a dependable and non-disruptive tag for studying protein localization in a wide variety of cell types (Stauber et al., 1998). While we must consider the possibility that the GFP tag might interfere with targeting, we consider this unlikely since GFP alone, or fused to TLTF polypeptides which lack the targeting domain, is abundantly expressed in the cytoplasm (Figs 1 and 2). The same is true for several mutant proteins in which only a single amino acid is changed within the targeting domain (Table 1). Furthermore, two mutant proteins (Fig. 5e and f), as well as the human-GFP fusion protein (Fig. 7b) exhibit highly restricted subcellular distributions that are not in the flagellar pocket region. Therefore, localization of the wild-type fusion protein is not simply the result of expressing a heterologous fusion protein. Although the flagellar pocket localization of TLTF might be considered consistent with that of a potentially secreted protein, we have not yet confirmed an earlier report that TLTF is released as a soluble factor from trypanosomes (Olsson et al., 1991), and we are working to test this possibility.

In our mutant screen, we expected to uncover mutations that either inhibited TLTF targeting or did not, and were surprised to isolate two mutant proteins (K191I and K191N/E196G) in which GFP is misdirected into the **flagellum** itself, rather than simply remaining in the cytoplasm as occurs for most other mutants (Figs 1, 2 and 5). As seen in Fig. 4A, the flagellar pocket region is the only area of the cell where the lumen of the flagellum is contiguous with the cytoplasm. This

organization presents a potential explanation for the phenotype of the K191I and K191N/E196G mutants (Fig. 8). Namely, these mutant proteins might retain enough targeting information to arrive in the general vicinity of the flagellar pocket but, once there, they miss an important sorting/ retention signal and are diverted into the flagellum, perhaps along an existing pathway for transport of endogenous flagellar proteins. An alternative possibility is that mutation of K191 generates a new binding site for an existing flagellum targeting pathway that is dominant over interactions that ordinarily retain the protein at the flagellar pocket. Such an 'intra-flagellar' protein transport pathway must exist in order to transport flagellar proteins, e.g. the paraflagellar rod proteins PFR-A and PFR-C, into the flagellum (Bastin et al., 1998). Interestingly, when the *T. brucei* PFR-A gene is deleted, the PFR-C protein still enters the flagellum, but abberantly accumulates at the free end of the flagellum, often leading to a swollen tip that is strikingly similar to the swollen tip in our K191 mutants (e.g. compare Fig. 5f with figure 2 of Bastin et al., 1998). A good candidate for an existing intra-flagellar transport pathway would be the flagellar microtubules, since microtubule-dependent transport mechanisms operate in cytoplasmic, axonal and intra-flagellar transport in other organisms (Hirokawa, 1998; Kozminski et al., 1995; Rosenbaum et al., 1999). Furthermore, intra-flagellar transport proteins in *Chlamydomonas reinhardtii* are often found near flagellar basal bodies (Rosenbaum et al., 1999), which in trypanosomes are located near the flagellar pocket. In any case, our data suggest that specific protein-protein interactions are critical for determining the ultimate destination of TLTF and that amino acids K191-E196 might be a binding site for other trypanosomal proteins or a specialized feature of the flagellar pocket membrane.

Like the K191 mutant proteins discussed above, the posterior location of the human-GFP fusion protein (Fig. 7b) provides insight into the mechanism of TLTF targeting when considered in the context of the trypanosome's cellular architecture. In *T. brucei*, the polarized subpellicular microtubules are uniformly oriented with their plus ends at the posterior of the cell (Robinson et al., 1995), corresponding exactly to the position of the human-GFP fusion protein. The only other protein known to be localized to this position is Gb4, a *T. brucei* plus-end microtubule capping protein (Rindisbacher et al., 1993). In this light it is worth noting that flagellar microtubules have exactly the opposite polarity to the subpellicular microtubules, their plus ends being at the tip of the flagellum (Robinson et al., 1995). Thus, it is perhaps no coincidence that the flagellum-targeted TLTF-GFP mutants sometimes accumulate at the tip ('plus-end') of the flagellum (e.g. Fig. 5f).

Together, our results lead us to propose a model (Fig. 8) for TLTF targeting that invokes involvement of the trypanosome cytoskeleton. In this model, microtubules serve as tracks for targeting of wild-type TLTF-GFP to the flagellar pocket region, where a conformational signal in TLTF recognizes a unique feature of the anterior flagellar pocket membrane (protein or lipid), leading to retention. Whether or not microtubule interactions continue to participate in maintaining the protein at this position remains to be determined. Given that TLTF does not contain an N-terminal leader sequence (von Heijne, 1982, 1983), we suggest that this targeting pathway is not through the classical, ER/golgi secretory pathway. Cytoplasmic mutant proteins loose all targeting information. The trypanosome K191 mutant proteins and the human protein miss the flagellar pocket 'retention signal' and continue along microtubule tracks in the plus end direction. The trypanosome K191 mutant proteins enter the flagellum, while the human protein goes to the cell posterior. This might be explained if the K191 mutant proteins exhibit a particular affinity to an existing intra-flagellar transport pathway, or retain a weak affinity to the flagellar pocket, bringing them into proximity with an intra-flagellar transport apparatus.

Microtubules are involved in protein targeting in a variety of organisms, including trypanosomes (Snapp and Landfear, 1997), and preliminary examination of TLTF in detergent-extracted cytoskeletons is consistent with microtubule involvement in TLTF targeting (K. L. Hill, N. R. Hutchings and J. E. Donelson, unpublished observation). There are four specialized, membrane-associated microtubules that terminate on the anterior side of the flagellar pocket (Vickerman and Preston, 1976), the same position where wild-type HI-GFP is located (Fig. 4A). Perhaps these microtubules play a special role in protein targeting to the flagellar pocket. We are only just beginning to appreciate the complexity of macromolecular trafficking in trypanosomes. The unique requirements of protein trafficking in these early diverging eukaryotes make it likely that efforts to understand these events will be rewarded with novel insights into fundamental questions in cell biology.

We would like to thank E. Ullu for the YTAT1.1 trypanosome clone, Brendan Cormack and Stanley Falkow for the GFPmut3 cDNA, and Christine Clayton for the pHD1 and pHD496 trypanosome expression vectors. We also thank Charles Lee for assisting with cloning of TLTF deletion fragments, and Dr Rachelle Crosbie for critical reading of the manuscript. We are grateful to Tom Moninger and the University of Iowa Central Microscopy Research Facility for guidance in the use of the confocal microscope. We acknowledge the support of the University of Iowa Diabetes and Endocrinology Research Center (Grant DK25295). D. G. R. is supported by PHS grant AI 37977 and is a recipient of the Burroughs Wellcome Scholar Award in Molecular Parasitology. This work was funded in part by NIH NRSAs (AI07511 and AI09872) to K. L. Hill and NIH Grant AI40591 to J. E. Donelson.

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