Characterization of a Targeting Motif for a Flagellar Membrane Protein in *Leishmania enriettii*

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The surface membranes of eukaryotic flagella and cilia are contiguous with the plasma membrane. Despite the absence of obvious physical structures that could form a barrier between the two membrane domains, the lipid and protein compositions of flagella and cilia are distinct from the rest of the cell surface membrane. We have exploited a flagellar glucose transporter from the parasitic protozoan *Leishmania enriettii* as a model system to characterize the first targeting motif for a flagellar membrane protein in any eukaryotic organism. In this study, we demonstrate that the flagellar membrane-targeting motif is recognized by several species of *Leishmania*. Previously, we demonstrated that the 130 amino acid NH2-terminal cytoplasmic domain of isoform 1 glucose transporter was sufficient to target a nonflagellar integral membrane protein into the flagellar membrane. We have now determined that an essential flagellar targeting signal is located between amino acids 20 and 35 of the NH2-terminal domain. We have further analyzed the role of specific amino acids in this region by alanine replacement mutagenesis and determined that single amino acid substitutions did not abrogate targeting to the flagellar membrane. However, individual mutations located within a cluster of five contiguous amino acids, RTGTT, conferred differences in the degree of targeting to the flagellar membrane and the flagellar pocket, implying a role for these residues in the mechanism of flagellar trafficking.

Eukaryotic flagella and cilia are present throughout the evolution of eukaryotic cells from early flagellated protozoa to mammalian cilia. These specialized organelles contain an internal microtubule-based axoneme that provides structure and generates movement (1, 2). Flagella and cilia play roles in cellular locomotion, trapping of food, and sensory functions (3). To carry out sensory functions, ciliary and flagellar membranes contain proteins unique to these organelles. For example, *Caenorhabditis elegans* expresses ODR-10 (a seven-transmembrane domain receptor), OSM-9 (a cation channel), and ODR-3 (a G protein) that localize to the cilia of AWA olfactory neurons (4–7). Mammalian ependymal cells, oviduct, and trachea contain a G protein subtype (Gq) that localizes predominantly in cilia (8). In addition to distinct protein compositions, considerable evidence has accumulated that flagella and cilia exhibit lipid compositions distinct from other domains of the plasma membrane (9). For example, *Paramecium* cilia contain 1.5 times more sterols and 5 times more sphingolipids relative to the lipid composition of the whole cell (9). Despite lipid and protein compositions distinct from the rest of the plasma membrane, the membranes of these organelles are contiguous with the plasma membrane. How these membrane subdomains are generated is a question of general importance in cell biology.

Previously, several motile processes have been observed that could be responsible for movement of proteins into and within the flagellar membrane (3, 10, 11). Furthermore, Dwyer et al. (7) have identified two proteins, ODR-4 and ODR-8, in *C. elegans* that are required for the localization of ODR-10 to the cilia of AWA olfactory neurons. However, little is known about the process by which specific membrane proteins are targeted to ciliary and flagellar membranes. To better study the mechanisms that generate the differential protein distributions in eukaryotic flagella, we have characterized a targeting motif for a flagellar integral membrane protein in the parasitic protozoan *Leishmania enriettii*.

*Leishmania* are single-cell primitive eukaryotes that have a plasma membrane that can be subdivided into three distinct subdomains: (a) flagellar, (b) flagellar pocket, and (c) pellicular plasma membranes (12) (Fig. 1A). Each subdomain has distinct protein populations (12). We have shown previously (13) that two closely related isoforms of the major glucose transporter from *L. enriettii* are differentially localized. Isoform 1 glucose transporter (ISO1) is located primarily in the flagellar membrane, whereas isoform 2 glucose transporter (ISO2) localizes primarily to the pellicular plasma membrane surrounding the cell body. Both isoforms are also present in the flagellar pocket membrane, the functionally specialized domain of the plasma membrane that receives proteins that are ultimately destined for the pellicular and flagellar membranes or secreted into the extracellular medium (12, 14, 15).

ISO1 and ISO2 differ only in the sequence of their cytosolic NH2-terminal domains; ISO1 has a 130-amino acid domain that lacks similarity to the 48-amino acid NH2-terminal domain of ISO2. We have previously demonstrated that the NH2-terminal domain of ISO1 is sufficient to target an integral pellicular plasma membrane protein to the flagellar membrane (16). In this study, we demonstrate that several species of *Leishmania* target ISO1 to the flagellar membrane, indicating that the ISO1 flagellar membrane-targeting sequence is broadly recognized among members of this genus. Based on the combined results of NH2-terminal deletions (16) and chimeric
Flagellar Membrane-targeting Motif

Several Leishmania Species Are Capable of Targeting ISO1 to the Flagellar Membrane—We have previously determined that the parasitic protozoan L. enriettii targets the glucose transporter ISO1 to the flagellar membrane (13). The genus

constructs, a critical flagellar targeting sequence has now been mapped to the region between amino acids 20 and 35. We have used alanine scanning mutagenesis of this region to identify a cluster of five contiguous amino acids that are likely to play a role in flagellar targeting.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection—Promastigotes of L. enriettii were cultured at 26°C in Dulbecco’s Modified Eagle-Leischmania medium (Life Technologies, Inc.) (17) containing 5% fetal calf serum and 5% bovine embryonic fluid or RPMI 1640 medium (Life Technologies, Inc.) containing 10% fetal calf serum. Both types of medium contained xanthine (0.1 mM) and hemin (50 μg/ml) supplements. Parasites were transfected by electroporation (18) of plasmid DNA using a Gene Pulser (Bio-Rad) apparatus. One day after transfection, hygromycin (Calbiochem-Novabiochem) was added to a final concentration of 50 μg/ml to the culture, and the parasites were maintained under these conditions until drug-resistant parasites grew out (usually 3–4 weeks). The hygromycin concentration was then increased to 200 μg/ml for maintenance of transfected parasites.

Antibody Preparation—Polyclonal antisera against a glutathione S-transferase fusion protein containing the 25 COOH-terminal amino acids of rat GLUT2 was purchased from Biogenesis. The antibody was used undiluted for immunofluorescence observation.

For immunofluorescence imaging, parasites were pelleted, washed twice in PBS, resuspended at a density of approximately 10^7 cells/ml, and attached to poly-L-lysine-coated coverslips. The adherent parasites were fixed with 100% methanol at −20°C for 15 min. After fixation, coverslips were rinsed in PBS and then incubated in PBS plus 2% goat serum for 15 min. Antiserum was added at the appropriate dilution in PBS plus 2% goat serum and incubated for 1 h at room temperature. Coverslips were rinsed three times in PBS and then incubated for 1 h with a 1:200 dilution of goat anti-rabbit IgG coupled to fluorescein isothiocyanate (Sigma) or to Texas Red (1:800) (Molecular Probes, Inc.) in PBS plus 2% goat serum. Coverslips were rinsed five times with PBS and then mounted onto slides in 50% Tris, pH 8.0, 90% glycerol, and 20 mg/ml n-propyl-gallate (Sigma).

Images were acquired at the Oregon Hearing Research Center of the Oregon Health Sciences University using a Bio-Rad MRC 1024 ES laser scanning confocal imaging system attached to an inverted Nikon Eclipse TE300 microscope with a ×60 oil immersion lens and a ×2 zoom. The acquisition system (LaserSharp) used a krypton/argon laser with excitation lines at 488, 568, and 647 nm and simultaneous or sequential detection using one/two/three 8-bit photomultiplier tubes. Images were processed using the LaserSharp (Bio-Rad) post-processing software.

Construction of Chimeras between ISO1 and ISO2—The plasmid pX63 HYG (19) was used for expression of epitope-tagged constructs in L. enriettii. The 13 COOH-terminal amino acids of rat GLUT2 to the flagellar membrane (13). The genus

to amino acid 4 of ISO2. Subsequently, the BamHI (P/Clai) fragment of this chimeric construct was cloned into the BglII (P/Clai) sites of the pX63 HYG ISO1 vector containing the ISO1 open reading frame fused to GLUT2 epitope tag (16), using the internal Clai site and the BglII site in the upstream polylinker region. This strategy generated chimeric constructs lacking a fragment of V-region, we combined the BamHI site and amino acid 4 of ISO2 and containing the 13-amino acid GLUT2 epitope tag at the COOH terminus. The regions containing the ISO1 fragment and its junction with ISO2 were sequenced, as reported previously (22), to confirm that the correct constructs had been generated. The ISO1 (1–35)::ISO2 construct was generated by cloning the BamHI (P/Asp/ Clai) fragment containing the ISO1 coding amino acids 1–35 into the BamHI (P/Asp) sites of pBS ISO2 EcoRI (P/Clai) (removing the first three amino acids of ISO2 (MSD)). The BamHI (P/Clai) fragment containing the chimeric region was then cloned into the pX63 HYG ISO1 BglII (P/Clai) sites as described above.

Alanine Replacement Mutagenesis of the ISO1 NH2-terminal Cytoplasmic Domain—Alanine scanning mutagenesis and the addition of new restriction sites to ISO1 were accomplished using a variation of the Promega Altered Sites II Mutagenesis Systems protocol. Briefly, mutagenic oligomers (sense orientation) containing both a single mutated codon to convert the encoded amino acid to an alanine and a translationally silent change of one or two wobble bases to add or remove a restriction site for ease of screening were phosphorylated with the T4 polynucleotide kinase (Promega) and annealed to a 1:2000 dilution of plasmid DNA from a Bluescript-based plasmid containing a fragment of ISO1 (bases 1–1432 of the open reading frame terminating in the single Clai site within the open reading frame) in the antisense orientation (23). DNA synthesis and ligation were performed, and the product was transformed into ESI301, a mutS strain of Escherichia coli, and then into XL-1 blue bacteria as described previously (24). Mutants were screened by restriction digests, followed by sequencing of the mutated DNA, as described below. Mutagenized constructs were excised with BamHI (P) and Clai and ligated into the BglII (P)/Clai site of the pX63 HYG T230 vector (16), which produced an expression vector encoding the mutant ISO1 protein containing a 13-amino acid epitope tag from rat GLUT2 at the COOH terminus (see below).

Statistical Analysis of Flagellar Membrane-targeting Phenotypes—To characterize flagellar membrane-targeting phenotypes, 30 different confocal immunofluorescence images of each wild-type or mutant ISO1-expressing cell line were obtained. Each 512 × 512, 72 pixel/μm image was analyzed using NIH Image 1.61 for Power PC Macintosh on a Power Macintosh 7300. Files were opened as gray scale images and inverted (i.e., bright pixels become gray to black, and dark pixels become white) for ease of processing. The different plasma membrane subdomains (flagellar, flagellar pocket, and pellicular plasma membranes) are illustrated in Fig. 1A. The flagellar pocket was designated as the most intense 3 × 3-pixel area inside the cell within 6 pixels of the point at which the flagellum and pellicular plasma membrane join. Flagellar fluorescence values were taken as the average of three 3 × 3-pixel areas along the flagellum (specifically, the area within 2 pixels in front of the area where the flagellum joins the cell body, a point approximately in the middle of the flagellum, and the tip of the flagellum) (Fig. 1B). Fluorescence values for flagella and the flagellar pockets were normalized to a scale of 1–100 for each cell, and the flagellar pocket value was designated as 100. The relative flagellar intensity was defined as the average of the three flagellar fluorescence values divided by the flagellar pocket fluorescence value (Fig. 1B). For wild-type ISO1-expressing cells and each mutant, a histogram of relative flagellar intensities for 30 cells was plotted. Because the histograms of these data did not fit a normal distribution, we used the χ² statistical test to determine whether differences in the relative flagellar fluorescence staining were significant between lines expressing the mutant or wild-type ISO1. Because no specific criteria exist to divide relative flagellar intensities into high and low values, we compared the significance of the relative distributions of flagellar intensities at two different cutoffs (80% and 60% of relative flagellar pocket intensity). In all cases where statistically significant differences were found, distinct mutant phenotypes differed significantly (p < 0.05) from the wild-type epitope-tagged ISO1 in the amount of staining that occurred above both the 80% and 60% cutoff values.

RESULTS

Several Leishmania Species Are Capable of Targeting ISO1 to the Flagellar Membrane—We have previously determined that the parasitic protozoan L. enriettii targets the glucose transporter ISO1 to the flagellar membrane (13). The genus
Mapping of the Flagellar Targeting Motif Using Chimeric Constructs

To define the flagellar membrane-targeting sequence of ISO1 more precisely, we prepared chimeric constructs that encode segments of the NH$_2$-terminal domain of ISO1 fused to amino acid 4 of ISO2 (Fig. 3A), a protein normally targeted to the pellicular plasma membrane (Fig. 3B). These constructs were transfected into Leishmania promastigotes, and the location of each chimeric protein was determined by confocal immunofluorescence microscopy. The data in Fig. 3C indicate that the chimaera containing amino acids 1–35 of ISO1 fused to the NH$_2$ terminus of ISO2 largely retargeted to the flagellar membrane, confirming that this region contains all of the essential flagellar targeting sequence. Furthermore, the previous observation that the first 20 amino acids of ISO1 could be deleted without affecting flagellar targeting (16) implies that the region between amino acids 20 and 35 (SSPPRTGTTSHAAHN; Fig. 4) encompasses all of the essential flagellar targeting information. A chimera containing amino acids 20–35 of ISO1 revealed a partial retargeting phenotype (Fig. 3D). Staining patterns among different cells in the population varied from primarily pellicular plasma membrane to predominantly flagellar membrane. The partial retargeting is reminiscent of the partial flagellar targeting of the 25-amino acid deletion of the ISO1 NH$_2$ terminus (16), which also varies for individual cells in a population. In contrast, chimaeras containing shorter segments of ISO1 (see “Experimental Procedures”) remained in the pellicular plasma membrane (data not shown), as did the wild-type ISO2 protein (Fig. 3B). Consequently, the shortest segment that was sufficient to cause complete flagellar retargeting, at least in the context of these particular chimaeras, contained amino acids 1–35, whereas amino acids 20–35 could induce partial retargeting.

Ala$_l$ne Replacement Mutagenesis—To identify which amino acids are required for flagellar targeting, we individually mutated amino acids 22–31 to alanine and amino acid 32 from alanine to glycine and used confocal immunofluorescence microscopy to determine the localization of each mutant protein.
The results (Fig. 4, A–C) indicate that no single alanine mutation retargeted ISO1 to the pellicular plasma membrane, implying that no single amino acid was essential for flagellar targeting. Many mutants (P22A, P23A, R24A, S30A, H31A, and A32G) had a staining pattern that was similar to that of the wild type (Fig. 4A). However, visual inspection of other images suggested that some mutants (G27A, T28A, and T29A) were not expressed as abundantly on the flagellar membrane as wild-type ISO1 but had a greater relative degree of staining in the region of the flagellar pocket (Fig. 4B). Furthermore, two mutants (R25A and T26A) stained almost exclusively on the flagellar membrane and not in the flagellar pocket (Fig. 4C).

To determine whether ISO1 was quantitatively redistributed on the surface membrane in any of the mutants, confocal immunofluorescence images were obtained for 30 cells for each mutant cell line. Intensities were measured for the flagellar pocket and flagellum as described under “Experimental Procedures.” The number of cells (of 30 cells) with flagella staining at least 80% as intensely and at least 60% as intensely as the flagellar pocket is listed in each column. The number of cells above the cutoff and below the cutoff was compared to the numbers for wild-type ISO1. Significance was determined by performing a χ² test for each set of comparisons. p values less than 0.05 were considered significant. The data for G27A, T28A, and T29A were determined to be significantly different from wild-type ISO1 for both cutoff values and are shown in bold.

| Mutant | Intensity > 80% flagellar pocket (p) | Intensity > 60% flagellar pocket (p) |
|--------|------------------------------------|------------------------------------|
| ISO1   | 14                                  | 25                                  |
| P22A   | 8 (0.18)                            | 18 (0.086)                          |
| P23A   | 9 (0.29)                            | 23 (0.75)                           |
| R24A   | 12 (0.79)                           | 20 (0.23)                           |
| G27A   | 0 (< 0.001)                         | 1 (< 0.001)                         |
| T28A   | 5 (0.026)                           | 16 (0.026)                          |
| T29A   | 1 (< 0.001)                         | 16 (0.026)                          |
| S30A   | 13 (0.61)                           | 21 (0.36)                           |
| H31A   | 6 (0.055)                           | 17 (0.049)                          |
| A32G   | 8 (0.18)                            | 18 (0.086)                          |

This table was assembled from the data in histograms as described under “Experimental Procedures.” The number of cells of (30 cells) with flagella staining at least 80% as intensely and at least 60% as intensely as the flagellar pocket is listed in each column. The number of cells above the cutoff and below the cutoff was compared to the numbers for wild-type ISO1. Significance was determined by performing a χ² test for each set of comparisons. p values less than 0.05 were considered significant. The data for G27A, T28A, and T29A were determined to be significantly different from wild-type ISO1 for both cutoff values and are shown in bold.
Identification of a Flagellar Targeting Motif in ISO1—In this study we have identified a targeting motif for a flagellar membrane protein in a eukaryotic cell. We have defined a minimal sequence, amino acids 1–35 of ISO1, that is capable of redirecting a pellicular plasma membrane protein, ISO2, to the flagellar membrane. Furthermore, our previous study demonstrated that the first 20 amino acids of ISO1 were not necessary for flagellar membrane localization (16). These data imply that an essential flagellar targeting motif maps between amino acids 20 and 35 of ISO1. The observation that the chimera containing amino acids 20–35 of ISO1 fused to ISO2 retargets to the flagellar membrane (Fig. 3D), albeit inefficiently, is consistent with this conclusion but indicates that the sequences surrounding amino acids 20–35 must also influence the ability of this region to function in flagellar targeting. Potential context requirements could include the distance of sequence from the first transmembrane domain, the charge and structure of the sequence between the motif and the first transmembrane domain, and the effects of surrounding sequence on motif folding and solvent exposure.

We performed alanine replacement scanning mutagenesis to identify amino acids between positions 22 and 32 that influence flagellar targeting. The inability of individual amino acid replacements to prevent targeting of ISO1 to the flagellar membrane indicates that the flagellar targeting sequence is not absolute and allows for amino acid substitutions. We attempted to further disrupt flagellar membrane targeting by creating double alanine replacement mutants for amino acids 24–29. Confocal immunofluorescence microscopy revealed that cells expressing these double mutants stained poorly, and no distinct localization could be determined. However, the ability of individual alanine replacement mutants clustered between amino acids 25 and 29 (RTGTT) to affect the distribution of ISO1 to the flagellar membrane and flagellar pocket strongly supports a role for these five amino acids in flagellar targeting. The fact that some mutations (R25A and T26A) promote a greater degree of targeting to the flagellar membrane than wild-type transporter, whereas other mutations (G27A, T28A, and T29A) provoke a greater degree of targeting to the flagellar pocket, suggests that each of these two classes of mutation may be affecting a different aspect of flagellar targeting. For instance, G27, T28, and T29 might be important for retrograde transport from the flagellum to the flagellar pocket.

DISCUSSION

Comparison to Other Trypanosomatid Flagellar Membrane Proteins—Leishmania species are members of the order Kinetoplastida (25), which contains other related flagellated parasites such as Trypanosoma cruzi and Trypanosoma brucei. Among these parasites, proteins that have been localized to the flagellar membrane include the flagellar calcium-binding proteins of T. cruzi (31, 32) and T. brucei (33) and a putative receptor adenylate cyclase from T. brucei, ESAG4 (34, 35). The regions of the T. brucei proteins that are required for flagellar targeting are unknown. The first 24 amino acids (MGACG-SKGSTSDKGLASDKDGKKA) of the T. cruzi flagellar calcium-binding protein are necessary and sufficient for flagellar membrane targeting, and the dual myristoylation/palmitoylation of this sequence is required for membrane association and hence for targeting to the flagellum (32). The only apparent similarity within this 24-amino acid sequence and amino acids 20–35 of ISO1 (Fig. 4D) is between amino acids 7–13 of flagellar calcium-binding protein (KGSTSDK) and amino acids 24–31 of ISO1 (RTGTTSH). Both sequences initiate and terminate with a basic residue and contain several hydroxyl amino acids. However, it is currently unclear whether these potential similarities are functionally significant or whether ISO1, a polytopic membrane protein, and flagellar calcium-binding protein, a non-integral membrane protein that is tethered to the cytoplasmic surface of the membrane by a dual fatty acid modification, utilize similar or different targeting machinery.

Potential Mechanisms for Addition and Retention of ISO1 in the Flagellar Membrane—One long term goal is to characterize the mechanism and cellular components that direct proteins to the flagellar pocket staining. Analysis by the Chou-Fasman algorithm (30) of the region including and surrounding the flagellar targeting motif suggests that amino acids 20–23 and 26–29 have a high propensity to form β-turns, raising the possibility that this structure or some other secondary structure is an important component of the recognition of the flagellar targeting sequence by the targeting machinery. Determining whether such structural motifs on the protein are important in the mechanism of flagellar targeting will require identification of possible interacting partners and structure determination of the hydrophilic NH2-terminal domain of ISO1.

Comparison of flagellar pocket staining and flagellar staining

| Mutant  | Positive flagellar pocket | Negative flagellar pocket (p) |
|---------|--------------------------|-------------------------------|
| ISO1    | 29                       | 1 (0.47)                      |
| P23A    | 29                       | 1 (0.47)                      |
| R25A    | 11                       | 19 (<0.001)                   |
| T26A    | 11                       | 16* (<0.001)                  |

* Three cell images were discarded because background staining obscured the flagellar pocket and the ability to score.

flagellar pocket staining could not be performed for mutants R25A and T26A because relatively few cells (11 of 30 and 11 of 27 cells for R25A and T26A, respectively) displayed significant flagellar pocket staining. However, the flagellar membranes for both mutants stained intensely and were analyzed using an alternative method. The numbers of cells with or without flagellar pocket staining were compared relative to wild-type ISO1 (Table II). The number of parasites lacking staining in the flagellar pocket was much greater for both R25A and T26A mutants than for wild-type ISO1 (p < 0.001). The results suggest that R25A and T26A either enter the flagellar membrane from the flagellar pocket more efficiently than wild-type ISO1 or are retained more efficiently in the flagellar membrane once they arrive there. Taken together, these results indicate that mutations in amino acids 25–29, RTGTT, significantly affect or are retained more efficiently in the flagellar membrane once they arrive there.

The targeting of ISO1 and suggest that these amino acids are mutations in amino acids 25–29, RTGTT, significantly affect or are retained more efficiently in the flagellar membrane once they arrive there. Taken together, these results indicate that mutations in amino acids 25–29, RTGTT, significantly affect or are retained more efficiently in the flagellar membrane once they arrive there. Taken together, these results indicate that mutations in amino acids 25–29, RTGTT, significantly affect or are retained more efficiently in the flagellar membrane once they arrive there.
and maintain the distinct protein composition of the flagellar membrane. Studies by Bloodgood (3), Kozminski et al. (10), and Rosenbaum et al. (11) describe two distinct forms of protein transport specific to the flagellum. Bloodgood described gliding, the bidirectional Ca\(^{2+}\)-dependent movement of polystyrene beads on the flagellar membrane of Chlamydomonas reinhardtii (3). Kozminski et al. (10) characterized intraflagellar transport, which involves bidirectional movement of “raft” structures between the axoneme and the flagellar membrane (10, 36) and may also be responsible for the movement of some integral membrane proteins in the flagellar membrane (11). Both types of movement are dependent upon a flagellar kinesin-like protein, FLA10 (36). This kinesin-like protein colocalizes with electron-dense “lollipop” structures in transmission electron micrographs of C. reinhardtii flagella. Studies in C. elegans and Drosophila melanogaster have characterized similar lollipop structures in cilia (11), which suggest that intraflagellar transport has been evolutionarily conserved. Although the mechanism of ISO1 transport to the flagellar membrane remains to be elucidated, it is possible that one of the two flagellar transport processes cited above could be involved in this trafficking event. The flagellar targeting motif will be a useful tool that should enable us to identify proteins that may recognize this sequence and ultimately form the machinery for flagellar membrane protein trafficking.

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