Differential Targeting of Two Glucose Transporters from *Leishmania enriettii* Is Mediated by an NH₂-terminal Domain

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Abstract. *Leishmania* are parasitic protozoa with two major stages in their life cycle: flagellated promastigotes that live in the gut of the insect vector and nonflagellated amastigotes that live inside the lysosomes of the vertebrate host macrophages. The Pro-1 glucose transporter of *L. enriettii* exists as two isoforms, iso-1 and iso-2, which are both expressed primarily in the promastigote stage of the life cycle. These two isoforms constitute modular structures: they differ exclusively and extensively in their NH₂-terminal hydrophilic domains, but the remainder of each isoform sequence is identical to that of the other. We have localized these glucose transporters within promastigotes by two approaches. In the first method, we have raised a polyclonal antibody against the COOH-terminal hydrophilic domain shared by both iso-1 and iso-2, and we have used this antibody to detect the transporters by confocal immunofluorescence microscopy and immunoelectron microscopy. The staining observed with this antibody occurs primarily on the plasma membrane and the membrane of the flagellar pocket, but there is also light staining on the flagellum. We have also localized each isoform separately by introducing an epitope tag into each protein sequence. These experiments demonstrate that iso-1, the minor isoform, resides primarily on the flagellar membrane, while iso-2, the major isoform, is located on the plasma membrane and the flagellar pocket. Hence, each isoform is differentially sorted, and the structural information for targeting each transporter isoform to its correct membrane address resides within the NH₂-terminal hydrophilic domain.

F ACILITATED glucose transporters are integral membrane proteins (31), present in a diverse spectrum of organisms from bacteria to humans, that shuttle glucose across the plasma membrane and allow uptake and subsequent metabolism of this vital nutrient. Many organisms possess multiple glucose transporter isoforms with distinct biochemical properties (35), and some of these isoforms are localized to different subcellular compartments. Thus, mammalian GLUT1 is targeted primarily to the plasma membrane in fat and muscle cells, whereas GLUT4 is directed primarily to intracellular vesicles (32) that fuse with the plasma membrane after insulin stimulation of the target cell. A problem of considerable interest has been to determine how such isoforms are differentially targeted to their respective subcellular locations.

Previous studies on the parasitic protozoan *Leishmania enriettii* have revealed that this microorganism expresses two closely related isoforms (34) of a major glucose transporter designated Pro-1. Both isoforms of the Pro-1 transporter are expressed in the promastigote or insect stage of the parasite life cycle, but their mRNAs accumulate to only a residual level in the amastigote form of the parasite that lives within the macrophage lysosomes of the vertebrate host (4). These two isoforms (iso-1 and iso-2) possess distinct NH₂-terminal hydrophilic domains, predicted to be oriented on the cytoplasmic side of the plasma membrane (11, 14), but the remainder of their sequence, including the 12 putative transmembrane segments, the connecting hydrophilic loops, and the COOH-terminal hydrophilic tail, is identical in both proteins (see Fig. 1). Each isoform has been functionally expressed in *Xenopus* oocytes and shown to transport the glucose analogue 2-deoxy-D-glucose (18). These expression studies reveal that the substrate specificities, pharmacological properties, and kinetic parameters of each isoform are very similar, demonstrating that the distinct NH₂-terminal domains do not significantly affect these functional properties. One other potential role for these unique domains could involve differential subcellular targeting of each isoform. To determine the overall subcellular distribution of the Pro-1 transporters, we have used a COOH-terminal specific anti-

body that recognizes both iso-1 and iso-2. We have also used epitope tagging (17) to differentiate between the two proteins and thus define the location of each individual isoform. The results of these experiments reveal that iso-1, the less abundant isoform, is targeted primarily to the flagellar membrane of the parasite, whereas iso-2, the major isoform, is targeted to the plasma membrane and to the flagellar pocket, an invagination of the plasma membrane located at the base of the flagellum. These results clearly implicate the NH_2-terminal domain of each glucose transporter as a determinant in the subcellular targeting process, since this is the only domain that differs between the two isoforms. These observations also suggest that specific targeting signals must exist in at least one of these two distinct NH_2-terminal sequences.

Materials and Methods

Plasmid Constructs

The plasmid pX63Hyg (10) was used for expression of epitope tagged constructs in *L. enriettii*. A DNA sequence encoding the 13 COOH-terminal amino acids of rat GLUT2 (36) (TVQMEFLGSSETV), which were used as the epitope tag, was introduced into iso-1- and iso-2-containing plasmids by polymerase chain reaction-based mutagenesis. For the iso-1 construct, a primer internal to the protein coding sequence (encoding amino acids VTTLASI and containing the unique ClaI site [4] of the Pro-1 insert) was used as the forward primer, and an antisense primer encoding the six COOH-terminal amino acids of Pro-1 (IGNRAE) followed by the 13 amino acids from GLUT2 and a BamHI site was used as a reverse primer to amplify the modified COOH-terminal coding region. This amplified fragment was gel purified, digested with ClaI and BamHI, and cloned into an iso-1-containing Bluescript plasmid between the ClaI site internal to the protein coding region and the BamHI site present in the plasmid polylinker. The region from this internal ClaI site to the modified COOH terminus was sequenced from single-stranded DNA using the Sequenase polymerase (United States Biochemical Corp., Cleveland, OH) and the manufacturer's instructions. The SmaI/BamHI insert of this plasmid was then cloned into the SmaI/BglII site of the pX63Hyg plasmid to generate the pX63Hyg.iso1 vector for expression of epitope tagged iso-1. A similar strategy was used to construct pX63Hyg.iso2 for expression of epitope tagged iso-2.

For primer directed amplifications, reactions contained 50 mM Tris-HCl, pH 9.0, 50 mM NaCl, 2.5 mM $MgCl_2$, 200 μM each deoxynucleotide triphosphate, 1 μM each oligonucleotide primer, 0.2 ng plasmid DNA as template, and 1 U of DNA polymerase from *Thermus flavus* (Promega Corp., Madison, WI). Reactions were performed on a DNA thermal cycler (Perkin-Elmer Corp., Norwalk, CT) using a 4-min preincubation at 96°C followed by enzyme addition and then 20 cycles using a 40-s denaturation step at 94°C, a 1-min annealing step at 45°C, and a 1-min extension step at 72°C, followed by a single incubation at 72°C for 7 min.

Cell Culture and Transfection

Promastigotes of *L. enriettii* were cultured at 27°C in DME-L medium (15) containing 5% fetal calf serum and 5% bovine embryonic fluid. Parasites were transfected by electroporation (16) of plasmid DNA using a Gene Pulser (Bio Rad Laboratories, Richmond, CA) apparatus. Cells were resuspended at a density of 10^8 per milliliter in electroporation buffer containing 21 mM Hepes, pH 7.5, 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, and 6 mM glucose. A 0.4-ml aliquot of cells was mixed with 50 μg of sterile plasmid DNA in a 0.2-cm electroporation cuvette and subjected to a single discharge at 450 V and 500 μF. The transfected cells were chilled on ice for 10 min, transferred to 4.5 ml of DME-L medium, and incubated for 1 d at 27°C. At this time, hygromycin (Calbiochem-Novabiochem Corp., La Jolla, CA) 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 ~4 wk). The hygromycin concentration was then increased to 200 μg/ml for maintenance of transfected parasites.

Antibody Preparation

For preparation of an antibody against the COOH-terminal hydrophilic domain of the Pro-1 transporters, a segment of DNA containing the 32 COOH-terminal amino acids of Pro-1 (4) was amplified by the polymerase chain reaction using primers containing terminal BamHI sites. This amplified fragment was cloned into the BamHI site of the pGEX-2T vector (33), and the resulting construct was transformed into the XL1-Blue strain (Stratagene Cloning Systems, La Jolla, CA) of *Escherichia coli* for expression of a glutathione S-transferase fusion protein (PlC protein). Lysates were prepared as described (33) from 1-liter cultures after induction for 3 h with 0.4 mM isopropyl β-D-thiogalactopyranoside. These lysates were purified by binding to a column of glutathione-Agarose (Sigma Chemical Co., St. Louis, MO) followed by elution with 5 mM reduced glutathione. The eluted fusion protein was analyzed for purity by SDS-PAGE.

Rabbits were immunized with subcutaneous injections of ~0.5 mg of purified fusion protein emulsified in Freund's complete adjuvant followed by boosts containing 0.5 mg fusion protein in Freund's incomplete adjuvant at 4, 6, and 8 wk after the initial injection. 2 wk after the third boost, serum was collected and purified as described (13) by binding and elution from a column of protein A immobilized on Sephadex CL-4B (Sigma Chemical Co.). Antibodies against glutathione S-transferase were then subtracted from this partially purified antiserum by two sequential passages over a column containing glutathione S-transferase covalently coupled to Affi-Gel 10 resin using the instructions provided by the supplier (bulletin 1085; Bio Rad Laboratories). Finally, this antiserum was affinity purified as described (13) by binding to a column of PlC fusion protein covalently coupled to Affi-Gel 10 followed by elution with 100 mM glycine, pH 2.5. Fractions were immediately neutralized in an equal volume of 1 M Tris-HCl, pH 8.0, and were dialyzed against PBS, pH 7.2. Affinity-purified antibody was aliquoted and stored at −20°C. This affinity-purified antiserum was typically used at a dilution of 1:50 for immunofluorescence and for development of protein blots.

Polyclonal antiserum against the a glutathionine S-transferase fusion protein containing the 25 COOH-terminal amino acids of a rat GLUT2 was purchased from East Acres Biologicals (Southbridge, MA), and it was used at a dilution of 1:1,000.

Cell Lysates and Protein Blots

For preparation of total cell lysates, parasites at a density of ~1-2 × 10^7/ml were pelleted, washed with PBS, resuspended in Laemmli sample buffer (29) to a density of 0.2-1 × 10^9 cells/ml and immediately heated to 65°C for 5 min. Samples containing 10 μl of lysate were reheated to 65°C for 3 min, loaded onto 10% SDS-polyacrylamide gels, separated by standard methods (29), and electroblotted onto a nitrocellulose membrane using a Mini Trans-Blot apparatus (Bio Rad Laboratories) according to the manufacturer's instructions. Blots were developed using the ECL chemiluminescence system (Amersham Corp., Arlington Heights, IL) and goat anti-rabbit IgG coupled to horseradish peroxidase (Boehringer Mannheim Biochemicals, Indianapolis, IN), as detailed in the manufacturers' instructions, and the developed blots were exposed to XAR-5 film (Eastman Kodak Co., Rochester, NY).

For preparation of salt-extracted membranes, 3 × 10^9 cells were pelleted and rinsed twice in HES buffer containing 20 mM Hepes, pH 7.5, 1 mM EDTA, 255 mM sucrose. The pelleted cells were resuspended in 5 ml HES buffer on ice, broken by sonication using 15 pulses of 3 s each, and were checked by phase contrast microscopy to monitor cell breakage. This lysate was diluted with 15 ml of 100 mM $NaHCO_3$, pH 10.6, to remove peripheral membrane proteins, and it was centrifuged in a rotor (60 Ti; Beckman Instruments, Inc., Fullerton, CA) at 35,000 rpm for 90 min. The pellet was resuspended in a Laemmli sample buffer to a concentration of 1 × 10^{10} cells/ml and heated to 60°C for 5 min.

Flagella were prepared by resuspending 2 × 10^9 cells in 10 ml of 20 mM Tris-HCl, pH 8.0, 250 mM sucrose, 3 mM $MgCl_2$ (buffer A) on ice, and shearing with 200 strokes of a Dounce homogenizer with a tight-fitting (0.001-in. clearance) pestle. This sheared preparation was layered over 20 ml of 20 mM Tris-HCl, pH 8.0, and 25% sucrose (buffer B), and it was centrifuged at 700 g for 10 min to pellet intact cells and cell bodies. The upper phase was removed, layered over another cushion of buffer B and centrifuged again as above. The upper phase was finally layered over another cushion of buffer B and centrifuged at 10,000 g for 20 min to pellet the flagella. This pellet was resuspended in 100 μl PBS and examined by phase contrast microscopy to confirm that the flagella were largely free of cell bodies. The concentration of flagella was estimated by counting these organelles on a hemacytometer grid, and the preparation was dissolved in Laemmli sample buffer as described above.

The experiments represented in Fig. 2 A involved probing protein blots with anti-PlC antibody that had been competed by incubation with either

glutathione S-transferase or with PlC fusion protein. For these experiments, anti-PlC antibody was diluted 1:50 into 3 ml of 5% powdered milk in 20 mM Tris-HCl, pH 7.6, 150 mM NaCl, and 1% Tween-20. Glutathione S-transferase or PlC fusion protein was added to a concentration of 0.67 μg/ml, and this mixture was incubated for 1 h at room temperature before application to the protein blot.

Immunofluorescence Microscopy

For immunofluorescence imaging, parasites were pelleted, washed twice in PBS, resuspended at a density of ~10^7 cells/ml, and attached to poly-L-lysine–coated cover slips. In most instances, the adherent parasites were fixed with 100% methanol at −20°C for 15 min. For the experiment shown in Fig. 3, the parasites were fixed in 4% paraformaldehyde, buffered with PBS to pH 7.4, by incubation for 15 min, followed by quenching and permeabilization in 50 mM glycine containing 2% Triton X-100 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 FITC (Tago, Inc., Burlingame, CA) in PBS plus 2% goat serum. Coverslips were rinsed four times with PBS and then mounted on slides in 50% glycerol/50% PBS containing 1% p-phenylene diamine (Sigma Chemical Co.). For peptide competition experiments (see Fig. 2), the anti-PlC antibody at a 1:50 dilution was incubated for 1 h with 10 μg/ml of either Pep1 or Pep2, the mixture was centrifuged for 10 min at top speed in an Eppendorf microcentrifuge (Brinkmann Instruments Inc., Westbury, NY), and this preincubated antibody was applied to the coverslips. The sequence of Pep1 is HPWDEERDGKKV-VAPAIGKKELSEESIGNRAE, and the sequence of Pep2 is FDGGEE-GRAELNPSE.

For confocal microscopy, samples were examined with a confocal laser scanning microscope (Leica Lasertechnik GmbH, Heidelberg, Germany) equipped with a microscope (Fluorovert-FU; E. Leitz, Inc., Rockleigh, NJ), an argon/krypton laser, a Leitz 63× oil immersion objective, a 488-nm excitation filter, and a 530-nm long-pass barrier filter. Images were stored on a Motorola 68040 computer using System OS9 2.4 software (Microware Systems Corp., Des Moines, IA).

Immunoelectron Microscopy

Leishmania promastigotes were processed for immunoelectron microscopy as described previously (26, 27). In brief cells were fixed in either 1% glutaraldehyde, or 4% formaldehyde/0.25% glutaraldehyde in Hepes saline, pH 7.0. The promastigotes were pelleted, embedded in gelatin, and infiltrated with 2.3 M sucrose/20% polyvinyl pyrrolidone in PBS. The blocks were trimmed, frozen, and sectioned with an RMC MT7/CR21 cryoultramicrotome, and probed with antibodies.

All labeling experiments were conducted, in parallel, with controls omitting the primary antibody, and in the presence of "nonimmune" rabbit IgG. These controls were consistently negative at the concentrations of gold-conjugated second antibodies used in these studies. The antibodies used in these experiments included anti-PlC antibody against the PlC fusion protein, as well as anti-GLUT2 antibody raised against a 13–amino acid peptide corresponding to the COOH-terminus of GLUT2.

Results

Preparation and Characterization of an Antibody Directed Against the COOH-terminal Domain of the Pro-1 Transporters

The Pro-1 genes are arranged in a tandem array of approximately eight open reading frames that encode two different glucose transporter isoforms. Approximately seven of these open reading frames encode identical isoforms designated iso-2 (predicted molecular mass ~60 kD), while the 5' most open reading frame encodes a different isoform, iso-1 (predicted molecular mass ~70 kD). These two isoforms differ only at the hydrophilic cytoplasmically oriented NH$_2$-terminus (Fig. 1). Iso-2 encodes the more abundant mRNA
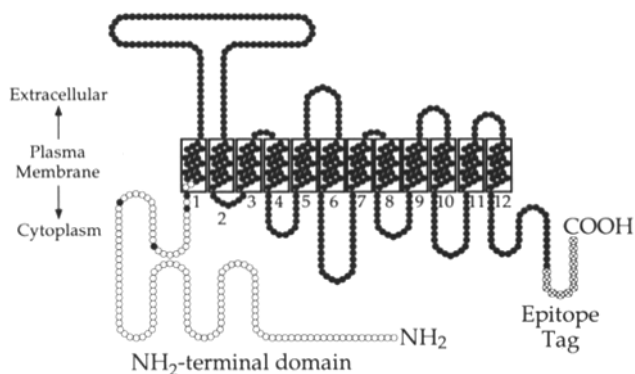

Figure 1. Schematic diagram showing the putative membrane topology of Pro-1 glucose transporters, the differences between iso-1 and iso-2, and the location of the epitope tag introduced for immunolocalization of each isoform. Black circles represent amino acids that are identical between the iso-1 and iso-2 isoforms of the Pro-1 transporter. Unfilled circles in the NH$_2$-terminal domain indicate amino acids that are different between the two isoforms. The structure shown in this figure is iso-1; iso-2 has a shorter NH$_2$-terminal domain (46 amino acids instead of the 130 amino acids present in iso1). The 13 circles filled with an X located at the COOH terminus represent the GLUT2 epitope tag that was introduced into both isoforms (see Materials and Methods). The numbered boxes designate putative transmembrane domains.

(34), and it would be expected to represent the majority of the Pro-1 transporters.

To obtain an antibody that recognized both iso-1 and iso-2, we prepared a recombinant fusion protein between glutathione S-transferase (33) and the 32 amino acids present in the COOH-terminal hydrophilic domains of the Pro-1 proteins (4). This fusion protein (here designated PlC) was used to immunize rabbits, and the resulting antisera were affinity purified. Immunoblotting of both total lysates of *L. enriettii* promastigotes and a salt-extracted particulate membrane fraction derived from these promastigotes was performed to demonstrate the specificity of the anti-PlC antibody. These blots (Fig. 2 *A*) reveal a band of ~50 kD apparent molecular mass in both the total and particulate lysates. The major Pro-1 transporter (iso-2) has a calculated molecular mass of 61.4 kD, but would be expected to migrate at a lower position on SDS-polyacrylamide gels since other highly hydrophobic proteins like mammalian glucose transporters run anomalously fast in this gel system (22). Furthermore, the blot in Fig. 2 *A* reveals that the ~50-kD band is effectively competed by the PlC fusion protein, but not by glutathione S-transferase. Hence, this antibody is specific for the COOH-terminal domain of the Pro-1 transporters.

Although there is a smear above the main band in Fig. 2 *A*, possibly caused by partial aggregation of monomers during the gel run, it is difficult to clearly discern a higher molecular mass band that could represent the larger (predicted 70.0 kD) iso-1 protein. This failure to detect a distinct band for iso-1 could be caused by the expected lower abundance of this protein compared to iso-2 and to the upward smearing of the iso-2 protein that would obscure such a lighter iso-1 band. However, results of epitope tagging experiments described below reveal that the iso-1 gene does encode a protein of ~70 kD that is localized primarily to the flagellum. To
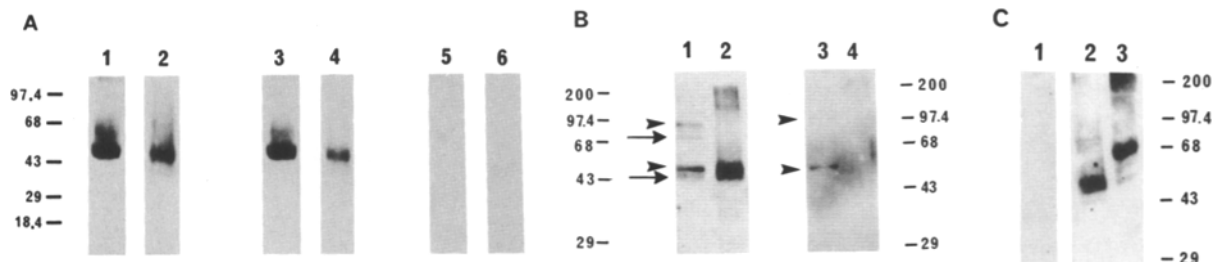
Figure 2. Protein blots of *L. enriettii* lysates probed with the affinity purified anti-P1C antibody (*A* and *B*) or the anti-GLUT2 antibody (*C*). (*A*) Samples of salt-extracted membrane pellets (lanes *1, 3,* and *5*) or total cell lysates (lanes *2, 4,* and *6*) were separated on a 10% SDS-polyacrylamide gel, blotted onto nitrocellulose, and probed with the anti-P1C antibody. Antibody incubations contained no competitor (lanes *1* and *2*), 0.67 μg/ml glutathione *S*-transferase (lanes *3* and *4*), or 0.67 μg/ml P1C fusion protein (lanes *5* and *6*). Numbers at the left indicate the mobilities of protein molecular mass markers in kilodaltons. (*B*) Samples of 3 × 10⁶ purified flagella (lanes *1* and *3*) and salt-extracted membrane pellets (lanes *2* and *4*) from 5 × 10⁶ cells were separated and blotted as above and probed with the anti-P1C antibody. For one blot (lanes *3* and *4*) the P1C antibody was preincubated with the Pep1 peptide (20 μg/ml) before application to the filter to block binding of antibody to Pro-1 polypeptides. The arrows indicate the two bands (∼50 and 70 kD) that are competed by pre-incubation with the Pep1 peptide, whereas the arrowheads indicate the two bands (∼50 and 90 kD) that are not competed by the Pep1 peptide. (*C*) Total lysates of *L. enriettii* transfected with the pX63Hyg parent plasmid (lane *1*), the pX63Hyg.iso2 expression plasmid (lane 2), or the pX63Hyg.iso1 expression plasmid (lane *3*) were separated on 10% SDS-polyacrylamide gels, transferred to nitrocellulose, and probed with the anti-GLUT2 antibody. Numbers indicate the mobilities of protein molecular mass markers in kilodaltons.

detect the unmodified version of the iso-1 protein, we purified flagella by shearing followed by differential centrifugation on sucrose gradients. An immunoblot of this flagellar fraction (Fig. 2 *B*, lanes *1* and *3*) probed with the P1C antibody clearly reveals a protein of ∼70 kD in addition to the ∼50-kD band previously identified in Fig. 2 *A* (Fig. 2 *B*, arrows). These two bands are specifically competed (Fig. 2 *B*, lane *3*) by a peptide (Pep1) representing the COOH-terminal hydrophilic domain of the Pro-1 proteins, confirming that they do represent Pro-1 polypeptides. The flagellar fraction also contains two other bands of ∼50 and ∼90 kD (Fig. 2 *B, arrowheads*) that are not competed by the Pep1 peptide and that may represent nonspecific binding of the P1C antibody to tubulins and paraflagellar rod proteins, respectively, the two most abundant classes of protein in the flagellum. Together, these results show that the P1C antibody reacts with the iso-1 and iso-2 isoforms of the Pro-1 polypeptides and that the iso-1 protein is enriched in the flagellar fraction.

The results presented in Fig. 2, *A* and *B*, were further confirmed by epitope-tagging experiments that distinguish between the two isoforms. An epitope tag representing the COOH-terminal 13 amino acids of rat GLUT2 (36) was introduced into iso-1 and iso-2 (Fig. 1). These modified constructs were subcloned into the extrachromosomal *Leishmania* expression vector pX63Hyg (10) and transfected into *L. enriettii* promastigotes. Immunoblots of extracts from the two resulting cell lines (Fig. 2 *C*) reveal that epitope-tagged iso-1 (pX63Hyg.iso1, lane *3*) and iso-2 (pX63Hyg.iso2, lane 2) are expressed at similar levels as polypeptides of ∼70 and ∼50 kD, respectively. No immunoreactivity was observed in whole-cell extracts prepared from promastigotes transfected with pX63Hyg alone (lane *1*).

Immunolocalization of Pro-1 Transporters Using an Antibody against the Pro-1 COOH Terminus

To localize the Pro-1 transporters, we have used the anti-P1C antibody in immunofluorescence and immunoelectron microscopy. Fixed promastigotes examined by confocal laser

scanning microscopy (Fig. 3) revealed staining over the body of the promastigote but concentrated at the periphery. This staining was also accompanied by a higher concentration of fluorescence over an oval-shaped body located at the base of the flagellum (intense fluorescence at upper left of parasite in Fig. 3, *A–C*, and labeled fp in Fig. 3 *B*). In one section of these confocal images (Fig. 3 *A*), it was also possible to detect light staining of the flagellum (*f*). Hence, these results suggest that the Pro-1 transporters are located at particularly high concentration in the flagellar pocket, an organelle known to contain receptors involved in endocytosis of nutrients in the related African trypanosomes (7, 8). These Pro-1 transporters also appear to be located on the plasma membrane over the entire surface of the cell body, and they may also be located at a low level on the flagellar membrane. The immunolabeling of all structures was specific since the labeling was completely abolished by the inclusion of the peptide Pep1 corresponding to the Pro-1 COOH terminus, but was unaltered by the inclusion of an irrelevant peptide, Pep2 (data not shown).

To confirm localizations obtained by confocal microscopy, we have also used the anti-P1C antibody in immunoelectron microscopy, detecting the primary antibody with secondary antibody labeled with gold particles (19). In these experiments, a small number of particles were visible (data not shown), and these particles were detected both on the plasma membrane and in the vicinity of the flagellar pocket, consistent with the results of confocal microscopy. A higher density of staining was obtained in subsequent experiments using epitope-tagged iso-2 that was overexpressed in parasites stably transfected with an iso-2–containing expression vector (see Fig. 6 below), as would be expected for cells expressing a higher level of transporter. However, it is notable that the qualitative location of gold particles in these epitope-tagging experiments was similar to the locations observed using the anti-P1C antibody to stain wild-type parasites.

Localization of Individual Isoforms by Epitope Tagging

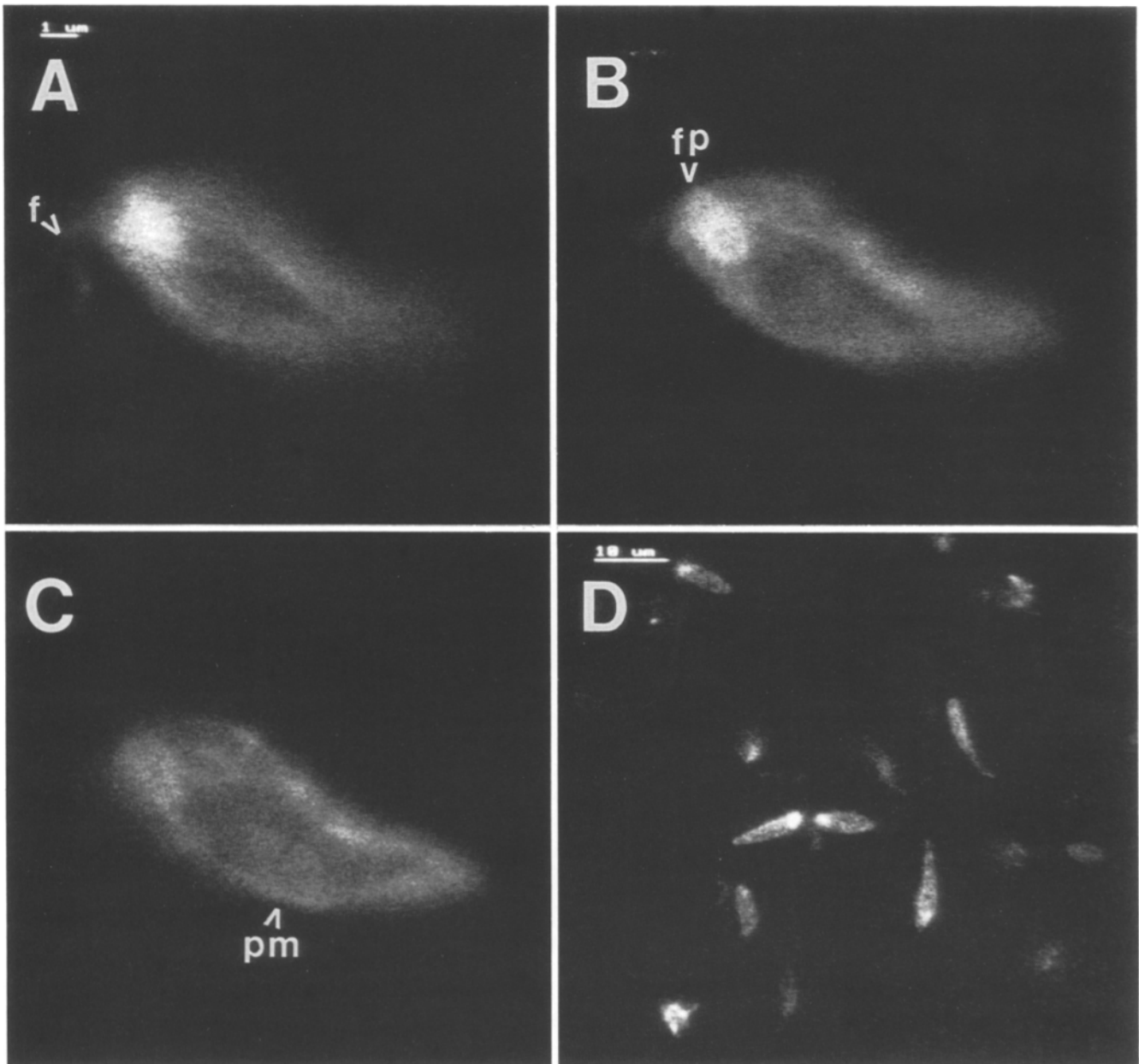Although the anti-P1C antibody can reveal the global local-

Figure 3. Confocal laser scanning micrographs of *L. enriettii* promastigotes stained with the anti-PIC antibody. Parasites were fixed with paraformaldehyde, stained with a 1:50 dilution of the anti-PIC antibody and then with an FITC-conjugated secondary antibody, and examined by confocal microscopy. (*A–C*) Three sequential 0.5-μm sections through a single parasite demonstrating staining of the plasma membrane (*pm*), flagellar pocket (*fp*), and flagellum (*f*). (*D*) A lower magnification showing staining of multiple parasites. Bar in *A*, 1 μm; bar in *D*, 10 μm.

ization of Pro-1 transporters, it can not distinguish between potential differential localization of iso-1 and iso-2 because the epitope that is recognized by this antibody is shared by both isoforms. Therefore, we used cell lines containing the epitope-tagged iso-1 and iso-2 constructs described above to determine the location if each isoform.

The differential localization of epitope-tagged iso-1 and iso-2 was clearly revealed by confocal microscopy (Fig. 4). Promastigotes transfected with pX63Hyg.iso2, encoding the epitope-tagged major isoform of Pro-1, showed intense staining at the periphery of the cell, representing the plasma membrane, and in the flagellar pocket (Fig. 4, *A* and *B*). In striking contrast, parasites transfected with pX63Hyg.iso1 displayed very intense staining on the flagellum with much lighter staining over the cell body (Fig. 4, *C* and *D*). This result reveals that iso-1 is targeted primarily to the flagellar membrane, while iso-2 is destined primarily for the plasma membrane and flagellar pocket. No labeling was detected in promastigotes transfected with vector alone (data not shown).

To confirm the differential localization of epitope-tagged iso-1 and iso-2 observed by immunofluorescence, we per-
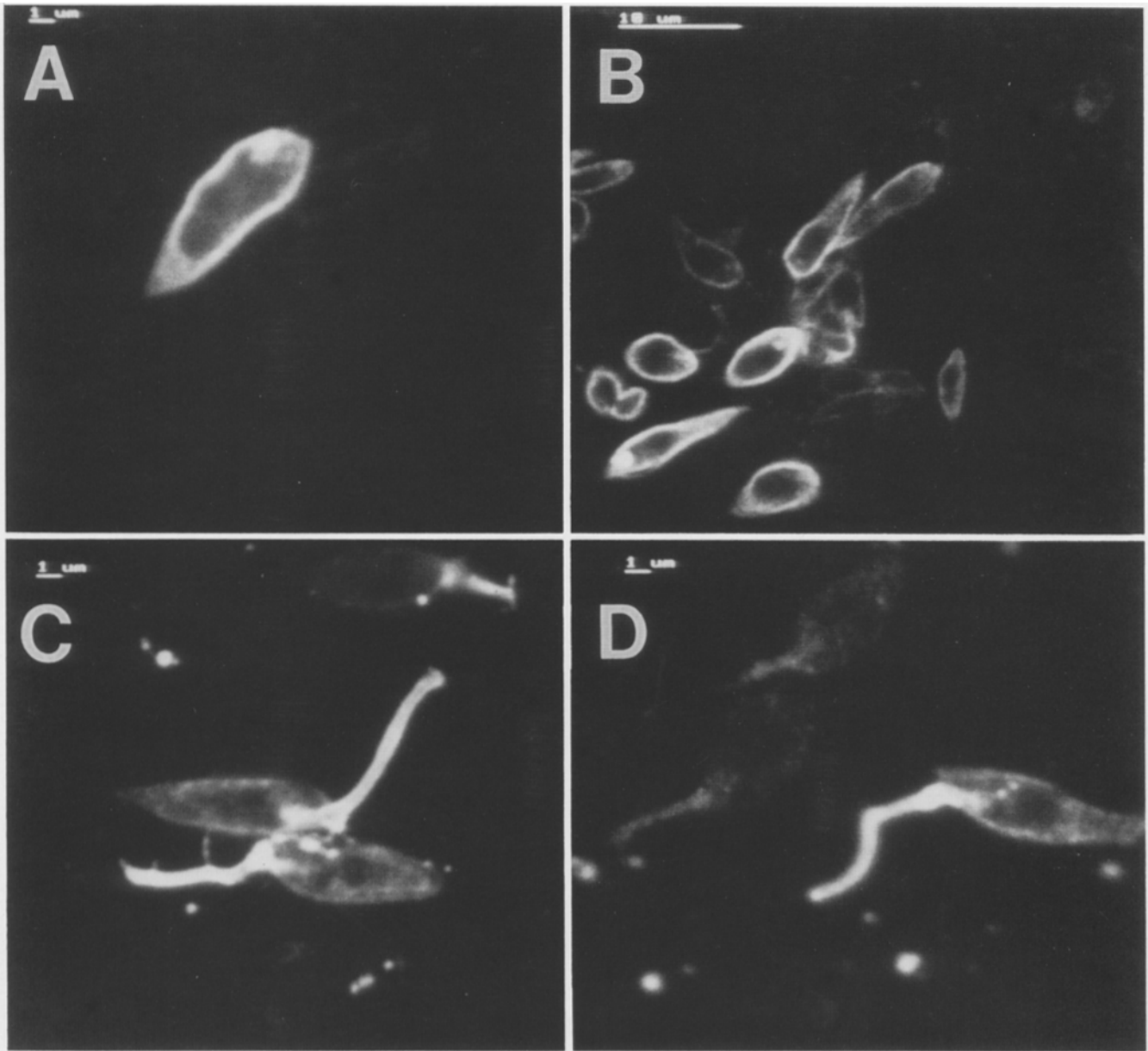
Figure 4. Confocal laser scanning microscopy of *L. enriettii* promastigotes expressing epitope tagged iso-1 or iso-2. Stable cell lines transfected with either pX63Hyg.iso2 (*A* and *B*) or pX63Hyg.iso1 (*C* and *D*) were stained with a 1:1,000 dilution of the anti GLUT2 antibody and an FITC-conjugated secondary antibody and examined by confocal microscopy. Each micrograph represents a single 0.5-μm section through each field. Bars in *A*, *C*, and *D*, 1 μm; bar in *B*, 10 μm.

formed immunoelectron microscopy on each of the transfected cell lines using the anti-GLUT2 antibody (Fig. 5 and 6). Cells transfected with pX63Hyg.iso1 were labeled strongly over the flagellar membrane (Fig. 5) and also over the plasma membrane and surrounding the flagellar pocket. Hence, although iso-1 appears to be targeted with a strong preference for the flagellar membrane, as revealed by the strong immunofluorescence signal over this organelle (Fig. 4, *C* and *D*), it is not localized exclusively in the flagellum. The immunofluorescence data, rather than the immunoelectron microscopy, probably provide a better quantitative estimate of the overall distribution of this isoform (i.e., strong preference for the flagellar membrane), since the immu-

nofluorescence image generates an integrated intensity over a larger area of the cell. Parasites transfected with pX63-Hyg.iso2 (Fig. 6) displayed gold particles primarily over the plasma membrane, but also occasionally at intracellular sites or over the membrane of the flagellar pocket. However, no staining was observed on the flagellar membrane.

As expected for cells that are overexpressing each protein, the strength of the signal for cells expressing either epitope-tagged construct and probed with the anti-GLUT2 antibody is considerably more pronounced than that observed with wild-type parasites stained with the anti-P1C antibody. However, we have not been able to quantitate the level of overexpression of these epitope-tagged proteins compared to the
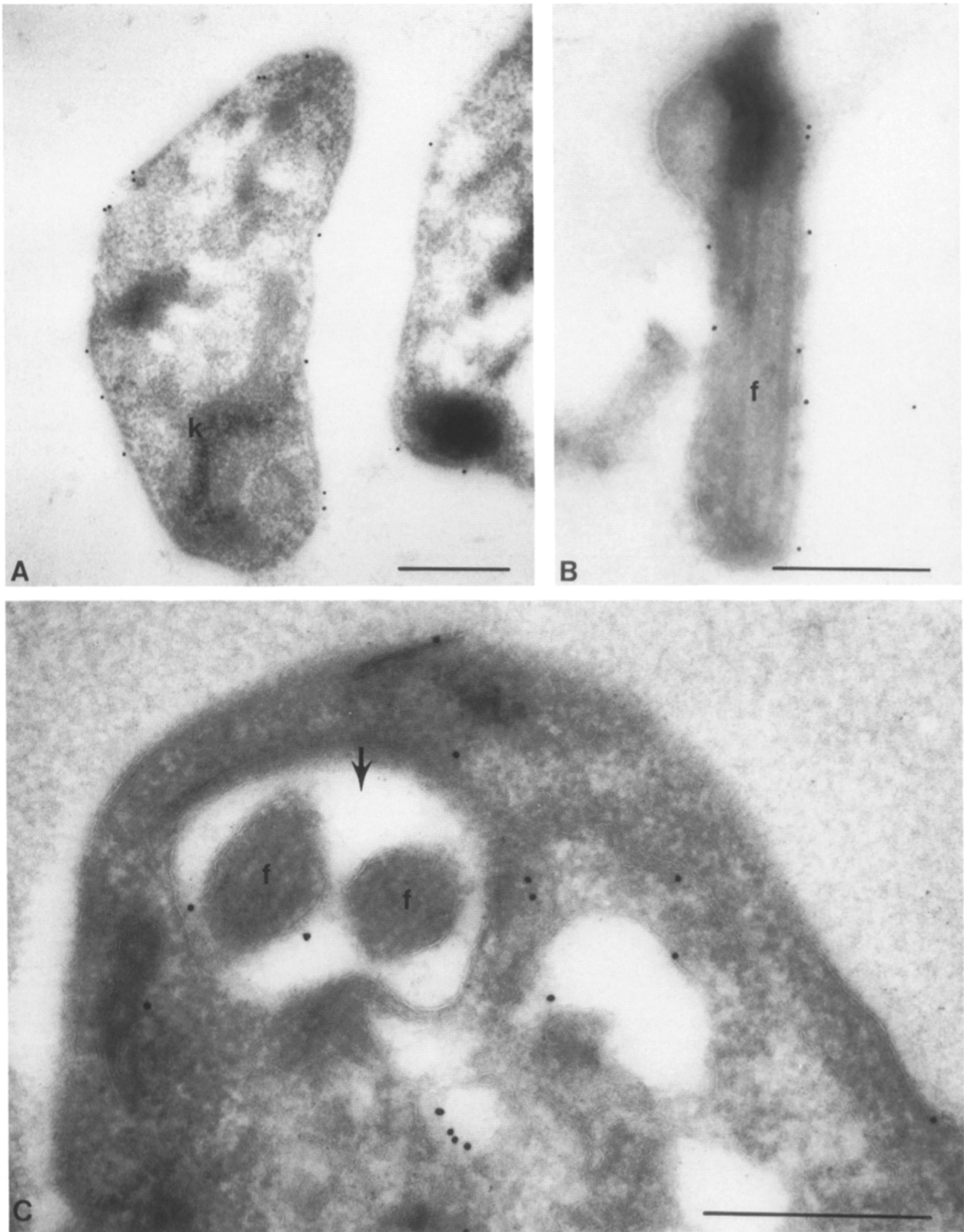
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Figure 5. Cryosections from *L. enriettii* promastigotes expressing iso-1 protein that has been epitope tagged with the GLUT2 COOH-terminal sequence. The sections were probed with rabbit anti-GLUT2 peptide and goat anti–rabbit IgG (15 nm gold). The antibody labeled clearly the promastigote plasmalemma (*A*). The promastigote kinetoplast is marked (*k*). The membrane around the flagellum (*f*) was also decorated by the antibody (*B*). The most intense labeling was found in the flagellar pocket (*arrow*) and in the vesicles that subtended the pocket (*C*). In each instance, the label was clearly membrane associated. Bars, 0.5 μm.
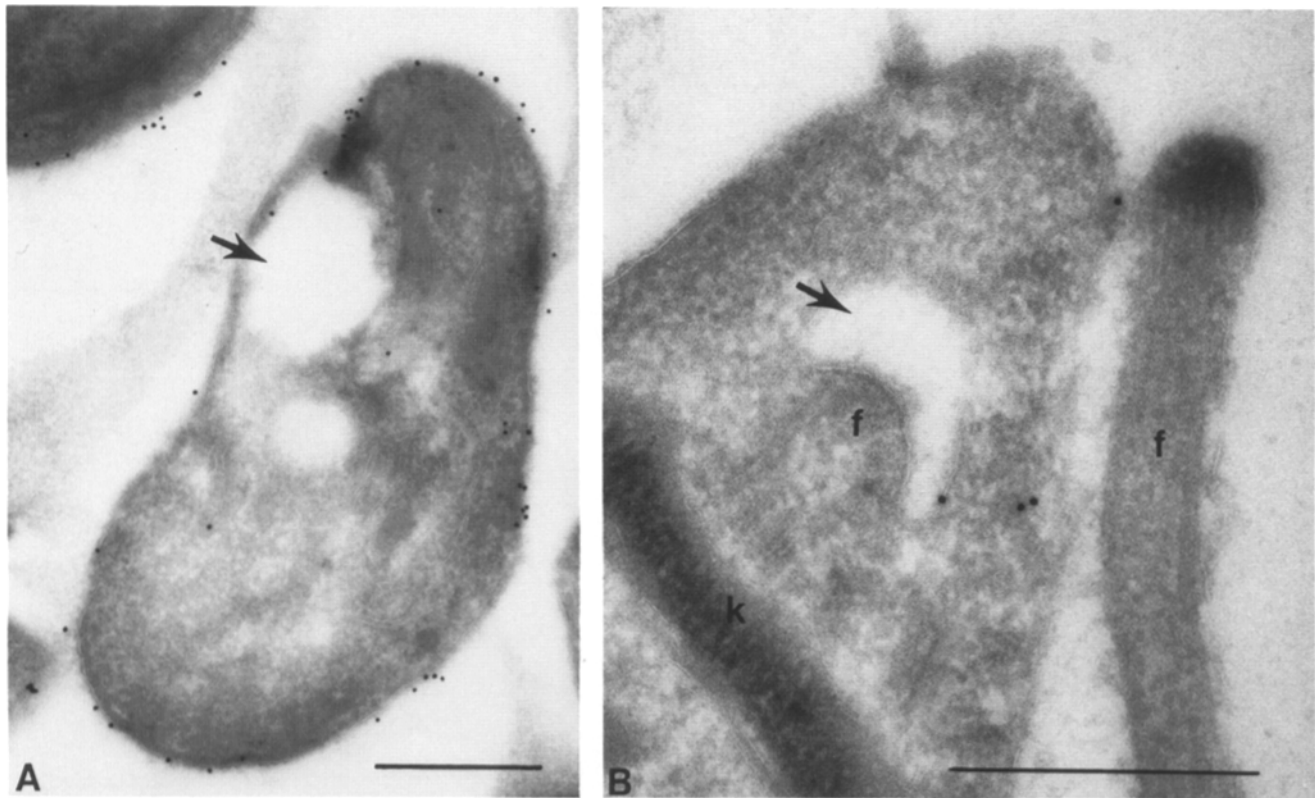
Figure 6. Cryosections from *L. enriettii* promastigotes expressing iso-2 protein that has been epitope tagged with the GLUT2 COOH-terminal sequence. The sections were probed with rabbit anti-GLUT2 peptide and goat anti–rabbit IgG (15 nm gold). Again, the antibody decorated the plasmalemma of the promastigote cell body (*A*). The labeling was also most intense in the flagellar pocket (*arrow*) region of the cells (*B*). However, in contrast to the iso-1 form that was present on the flagellar membrane, no label corresponding to the iso-2 form was found on the flagellar membrane. Bars, 0.5 μm.

wild-type transporters because the epitope tag apparently interferes with the ability of the anti-P1C antibody to react with the modified protein. Consequently, lysates from wild-type parasites and from the two transfectants generate bands of equal intensity on Western blots when probed with the anti-P1C antibody.

Discussion

Location of Membrane Proteins in Kinetoplastid Protozoa

There are still relatively limited data concerning the subcellular localization of integral membrane proteins in kinetoplastid protozoa such as *Leishmania* and *Trypanosoma*. A number of the more abundant surface proteins such as the variant surface glycoprotein (9), as well as the procyclin protein of *Trypanosoma brucei* (25) and GP63 (6) of *Leishmania* species have been shown to be present on the surface of the plasma membrane and on the flagellar membrane. These three proteins are tethered to the membrane via a glycosyl phosphotidylinositol anchor (1). A family of putative signal transduction receptors from *T. brucei* (24) are restricted to the flagellar membrane, suggesting a role for the flagellum in sensing the extracellular environment. Furthermore, the *T. brucei* receptors for low density liproprotein (7, 8), trans-

ferrin (8, 20, 28), and another trypanosome receptor (19) of unknown function are targeted to the membrane of the flagellar pocket, suggesting that this organelle may be specialized for uptake of nutrients. The flagellar pocket is thought to be the only site of receptor-mediated endocytosis in these parasites (37). However, it is currently unknown whether other classes of proteins involved in nutrient uptake, such as transporters, are also restricted to the flagellar pocket or distributed more broadly over the plasma membrane, as is the case for many mammalian transporters (36).

Our current studies on the *Leishmania* Pro-1 glucose transporters reveal that these proteins are located both in the flagellar pocket and on the plasma membrane and, hence, have a broader distribution that that observed for several proteins involved in receptor-mediated endocytosis. Thus, it appears that transport of glucose can occur across both regions of the plasma membrane. This difference between receptors and transporters may reflect the clustering of the cellular machinery involved in endocytosis beneath the membrane of the flagellar pocket (37), while transporters may be able to function in any membrane that is exposed to the exterior of the cell. Although we have not performed an extensive quantitative analysis, the apparent clustering of vesicles containing glucose transporters in the vicinity of the flagellar pocket (Fig. 5 *C*) suggests that this membrane may be a site for addition or removal of these proteins from the external membranes (37).

Differential Targeting of Glucose Transporter Isoforms to the Flagellar and Plasma Membranes

To define the subcellular localization of each isoform of the Pro-1 glucose transporters, we have engineered an epitope tag onto each protein, and have performed immunofluorescence and immunoelectron microscopy with an antibody against this epitope tag. One potential concern in any epitope-tagging strategy is that the protein of interest will be mistargeted by the alteration of the primary structure required for tagging. Another concern is that overexpression of the epitope-tagged transporters may result in their mislocalization. We believe these possibilities are unlikely. Both the iso-1 and iso-2 proteins have been tagged with the same epitope and overexpressed to similar levels (Fig. 2 C); hence, the only difference between these two tagged proteins is their distinct NH_2-terminal domains. Consequently, the differential targeting of these two labeled transporters must be caused by these different NH_2-terminal domains, and it cannot be explained by the incorrect routing of one isoform by the sequence modification or by overexpression. Furthermore, the localization of the epitope-tagged iso-2 is essentially identical to that observed for unmodified Pro-1 transporters, localized with the anti-P1C antibody. This is the expected result if this epitope-tagged isoform is directed in the same way as the major isoform present in wild-type parasites. Finally, we have demonstrated that the unmodified iso-1 protein is enriched in the flagellar fraction (Fig. 2 B); this result further supports the flagellar localization deduced from the epitope-tagging experiments. Collectively, these results confirm that this epitope-tagging strategy correctly identifies the distinct localization patterns for each glucose transporter isoform.

These studies lead to the compelling conclusion that the NH_2-terminal domains of these transporters are involved in their differential targeting, and that a major function of these distinct sequences must be to ensure the discrete routing of each isoform. This conclusion also implies that at least one of the two hydrophilic domains contains targeting signals that direct the protein to its correct membrane address. One possibility is that iso-2 follows a default pathway that ends in the plasma membrane and flagellar pocket, whereas iso-1 is actively sorted to be flagellar membrane via interactions with its NH_2-terminal domain. If this explanation is correct, the iso-1 NH_2 terminus may be able to redirect to the flagellar membrane chimeric constructs that contain this sequence attached to another transporter that normally resides in the plasma membrane. In addition, it may be possible to define specific targeting signals within these domains by mutagenizing this region of each isoform and observing potential alterations in the subcellular localizations. Studies of this type have defined internalization domains required for endocytosis of several mammalian receptors, including those for low density lipoprotein (12) and transferrin (5), and other consensus sequences have been implicated in the targeting of proteins to the Golgi membranes, endosomes, and lysosomes (21, 30). Finally, it is likely that such targeting domains interact with other proteins that direct one or both isoforms to their correct subcellular destinations. It may be possible to identify such protein–protein interactions by detecting polypeptides that physically interact with the NH_2-terminal domains.

One puzzle raised by the results presented here concerns the function of the flagellar glucose transporter. Since most of the glucose transporters expressed in the parasite are on the cell body, it is not clear why an apparently functionally identical transporter should be targeted to the relatively limited amount of membrane surface that covers the flagellum. It is unlikely that the flagellar location of one isoform could be explained by the requirement of the flagellum for an abundant source of ATP to be used for flagellar motility. Glucose transported across the flagellar membrane would first need to be translocated to the cytosolic glycosomes, the organelles that contain the glycolytic enzymes in kinetoplastid protozoa (23), before it could be metabolized to yield ATP. Although it is possible that a flagellar transporter could subserve some sensory role, it is not yet clear what this role might be, especially since Leishmania promastigotes apparently do not chemotax toward glucose (2). One possible approach to investigating the function of the flagellar isoform would be to perform a targeted gene replacement (10) to generate a null mutant of the iso-1 transporter. If there are any observable phenotypic differences in such an iso-1 null mutant, these defects might help to elucidate the specific function of this isoform.

One other glucose transporter family member that is located in flagella is the human fructose transporter GLUT5, which is present in both the plasma and flagellar membranes of spermatozoa (3). However, a major distinction between GLUT5 and iso-1 is that the human transporter is not differentially localized to either membrane. In contrast, it is clear that the flagellar membranes of kinetoplastic protozoa must differ significantly in composition from their plasma membranes since there are now at least two examples of integral membrane proteins, the iso-1 transporter of L. enriettii and the receptor-adenylate cyclases of T. brucei (24), that are localized primarily in the flagellar membrane. Further studies on the differential targeting of the iso-1 and iso-2 should help elucidate the mechanisms whereby these and possibly other proteins are sorted to the two distinct membranes.

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