Functional Expression and Subcellular Localization of a High-K_m Hexose Transporter from *Leishmania donovani*†

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ABSTRACT: We have used expression in *Xenopus* oocytes to characterize a new hexose transporter from the parasitic protozoan *Leishmania donovani*. This transporter utilizes the hexoses glucose, fructose, and mannose as substrates. A substrate saturation curve for 2-deoxy-D-glucose reveals a very high K_m, estimated to be approximately 150 mM. Immunolocalization of the protein with an antibody directed against the COOH terminus indicates that the transporter is present primarily in the parasite plasma membrane but is not detectable in the flagellar membrane. Since this protein is expressed in the insect stage promastigotes but not in the intracellular amastigotes, it may be specialized to function following an insect sugar meal when the concentrations of sugars surrounding the parasite are high.

Facilitative glucose transporters are integral membrane proteins that mediate the influx of glucose across the plasma membrane of many cells and are essential for the metabolic utilization of this important nutrient. These proteins constitute a family of structurally related polypeptides that are widely distributed throughout the plant and animal kingdoms (Baldwin & Henderson, 1989). Multiple glucose transporters are often expressed within a single species. These different subtypes have significant sequence similarity to each other but often exhibit functional differences including distinct substrate specificities, subcellular localizations, or kinetic parameters. Humans possess at least six facilitative glucose transporters that are specialized for different physiological roles (Mueckler, 1994). Thus, GLUT1 is a low-K_m transporter present in red blood cells and brain epithelia, GLUT2 is a high-K_m transporter that is thought to be involved in sensing blood glucose levels in pancreatic tissues, and GLUT5 is a fructose transporter located in intestinal epithelia and spermatozoa. Lower eukaryotes may also express a broad array of glucose transporters. The yeast *Saccharomyces cerevisiae* has at least seven functional glucose transporters which constitute two kinetically distinct systems, a high-affinity system with a K_m of approximately 1 mM and a low-affinity system with a K_m of approximately 20 mM (Ko et al., 1993).

The parasitic protozoan *Leishmania enriettii* possesses a family of hexose transporters that are encoded by the tandemly repeated Pro-1 genes (Stein et al., 1990). The first gene in this tandem repeat encodes an isoform that is targeted primarily to the flagellar membrane, whereas the other units of the repeat encode a closely related isoform that is sorted to the plasma membrane and flagellar pocket (Piper et al., 1995). Both isoforms have almost identical kinetic properties including K_ms for 2-deoxy-D-glucose (2-DOG)[1] that are approximately 0.5 mM (Langford et al., 1994). The mRNAs for both Pro-1 isoforms accumulate in the promastigote stage of the parasite that lives in the gut of the insect vector and which is exposed to sandfly sugar meals (Schlein, 1986), but these mRNAs are present at only residual levels in the amastigote stage of the life cycle (Cairns et al., 1989; Stack et al., 1990), when the parasite lives inside the phagolysosomes of the vertebrate host macrophages.

The gene for another putative transporter, called D2, has been cloned from *Leishmania donovani*, sequenced, and shown to encode a protein with 45% amino acid identity to the Pro-1 proteins (Langford et al., 1992). However, the D2 gene, which is also expressed in promastigotes but not amastigotes, has not been functionally expressed previously; consequently, it is uncertain whether it encodes a functional glucose transporter. In this paper, we have expressed the D2 gene in *Xenopus* oocytes and determined the substrate specificity and kinetic properties of the D2 transporter. Like the Pro-1 transporters, D2 transports glucose, fructose, and mannose, but the K_m for 2-DOG is much higher than that for either Pro-1 isoform. Immunolocalization of the D2 polypeptide reveals that it resides primarily in the plasma membrane and is excluded from the flagellar membrane. These results demonstrate that *Leishmania* parasites, like higher eukaryotes, possess multiple glucose transporters with pronounced functional differences. Each of these transporter subtypes may be specialized for distinct physiological roles.

EXPERIMENTAL PROCEDURES

Materials. Radiolabeled compounds were purchased from Dupont-New England Nuclear Corpn. All other chemicals were reagent grade.

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[1] Abbreviations: 2-DOG, 2-deoxy-D-glucose; GST, glutathione S-transferase; DCCD, *N',N'*-dicyclohexylcarbodiimide; FCCP, carbonyl cyanide [*p*-(trifluoromethoxy)phenyl]hydrazone; PBS, phosphate-buffered saline; FITC, fluorescein isothiocyanate.

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Culture of Parasites, Preparation of Cell Lysates, and Immunoblots. Promastigotes of *L. donovani* clone DI700 were cultured in Dulbecco's modified Eagle's-L medium (Iovannisci & Ullman, 1983) at 27 °C. Cell lysates for immunoblots were prepared from parasites grown to (1.5−2) × 10^7 cells mL^{-1} which were pelleted, washed three times in PBS, and resuspended in Laemmli sample buffer (Sambrook et al., 1989) at a final concentration of 2 × 10^9 cells $mL.^{-1}$ Lysates were heated to 65 °C for 5 min immediately upon suspension and before electrophoresis. Lysates were separated on 10% acrylamide Laemmli gels, electroblotted onto nitrocellulose, incubated with antibody at a 1:1000 dilution, and developed by enhanced chemiluminescence as described previously (Piper et al., 1995).

Expression of D2 Transporter in Xenopus Oocytes and Transport Assays. The coding region of the D2 gene was subcloned into the *Bgl*II/*Bam*HI site of the pL2-5 oocyte expression vector (Arriza et al., 1993), and capped cRNA was prepared from this vector as previously described (Langford et al., 1994). Defolliculated stage V−VI *Xenopus* oocytes (Colville & Gould, 1994) were injected with 5 ng of capped RNA, incubated at 17 °C for 4−5 days, and assayed for ability to transport various radiolabeled substrates as detailed previously (Langford et al., 1994). Negative controls were performed using uninjected oocytes. For studies involving non-sugar inhibitors, oocytes were preincubated with each compound for 10 min before initiating the transport assay. Assays for inhibition utilized a 1 h incubation with radiolabeled substrate, whereas those for substrate saturation were performed for 10 min. Control experiments showed that transport of both 10 μM and 300 mM 2-DOG in oocytes injected with D2 RNA was linear over this 10 min incubation. The K_m values were estimated by fitting the substrate saturation curves to the Michaelis−Menten equation as described (Langford et al., 1994). Transport assays on DI700 promastigotes were performed as reported previously (Langford et al., 1994).

Generation of Parasite Cell Lines Overexpressing the D2 Transporter. A 2 kb *Sac*I fragment containing the coding region of the D2 gene (Langford et al., 1992) was subcloned into the *Sac*I site of the *Leishmania* expression vector pALT-Neo (Laban et al., 1990), the resulting construct was transfected into DI700 promastigotes by electroporation (Piper et al., 1995), and the neomycin resistant transfectants were selected by culturing in 200 μg mL^{-1} G418.

Preparation of D2C Antibody. A sequence of DNA encoding the 26 amino acid COOH terminal hydrophilic domain of the D2 transporter was amplified using the polymerase chain reaction as described (Piper et al., 1995). This amplified fragment was subcloned into the *Bam*HI site of the pGEX-2T expression vector (Smith & Johnson, 1988), resulting in a construct that encodes a glutathione *S*-transferase (GST) fusion protein, called D2-GST. This plasmid was checked by DNA sequencing to ensure that the correct construct had been obtained. The recombinant plasmid was transfected into the XL-1 Blue strain (Stratagene Cloning Systems) of *Escherischia coli*, and the D2-GST fusion protein was induced and purified as described (Piper et al., 1995). Rabbits were injected subcutaneously with 1 mg of fusion protein emulsified in Freund's complete adjuvant, followed 2 weeks later by a subcutaneous injection of 1 mg of fusion protein in Freund's incomplete adjuvant. At 4, 6, and 8 weeks following the initial injection, rabbits were boosted with 250 μg of fusion protein in PBS containing a 10 mg mL^{-1} suspension of $Al(OH)_3$. Serum was collected before the initial injection (preimmune) and 6, 8, and 11 weeks thereafter. Serum from the final bleed was purified by binding and elution (Piper et al., 1995) from protein A conjugated to Sepharose CL-4B resin (Sigma Chemical Co.). The eluate was further affinity-purified by binding and elution to an Affigel-15 (BioRad Laboratories) column containing covalently conjugated D2-GST protein. Antibodies were removed from the column by elution with 100 mM glycine, pH 2.5, followed by elution with triethylamine, pH 11.5. The eluate was concentrated by binding and eluting a second time from a protein A column, and this D2C antiserum was aliquoted and stored at −70 °C.

Confocal Immunofluorescence Microscopy. Parasites were adhered to poly-L-lysine-coated coverslips, fixed in 100% methanol at −20 °C for 15 min, and prepared for immunofluorescence using a 1:300 dilution of the D2C primary antibody and a FITC-coupled goat anti-rabbit IgG secondary antibody (Tago Inc.) as described (Piper et al., 1995). Immunofluorescence images were obtained from a Leica confocal laser scanning microscope (Leica Lasertechnik GmbH) equipped with a Leitz Fluorovert-FU microscope, an argon/krypton laser, a Leitz 63× oil immersion objective, a 488 nm excitation filter, and a 530 nm long pass barrier filter at a resolution of 1024 × 1024 lines. Images were stored on a Motorola 68040 computer using System OS9 2.4 software (Microware Systems Corp.). Images were photographed using a Focus Graphics Image Recorder Plus from a 24 bit display monitor at a resolution of 1280 × 1024 lines.

Immunoelectron Microscopy. L. donovani promastigotes were fixed in 4% paraformaldehyde and 0.1% glutaraldehyde in 100 mM Hepes buffer, pH 7.2, for 1 h at ambient temperature and then washed three times in 100 mM Hepes, pH 7.2. Fixed parasites were then infiltrated overnight in a mixture of polyvinylpyrrolidone, 3.25 g (Sigma Chemical Co.); 1.1 M sodium carbonate, 0.625 mL; and 2.1 M sucrose dissolved in PBS, 11.75 mL. The samples were frozen in liquid nitrogen, and ultrathin sections were cut on a Reichert Ultracut E ultramicrotome with an FC-4E frozen thin section attachment (Reichert-Jung Optische Werke AG). The sections were suspended on loops containing 2.1 M sucrose and deposited onto Formvar-coated, carbon-stabilized grids. All immunolabeling steps were carried out at room temperature as follows. The grids were incubated for 15 min in PBS containing 10% fetal calf serum, followed by 30 min in primary antibody diluted 1:10 in PBS containing 1% BSA and 10 mM glycine (Buffer A), followed by five washes (5 min each) in Buffer A. Control incubations were performed in parallel using preimmune serum at 1:10 dilution in place of primary antibody. Subsequently, each grid was incubated for 15 min with a 1:20 dilution of goat anti-rabbit IgG conjugated to 10 nm gold particles (Amersham Corp.), washed five times with Buffer A, and finally washed four times in distilled H_2O. The sections were embedded in methylcellulose containing 0.1−0.4% uranyl acetate and viewed with a Philips 301 transmission electron microscope.

RESULTS

Expression of the D2 Transporter Gene in Xenopus Oocytes: Substrate and Inhibitor Specificity. To determine

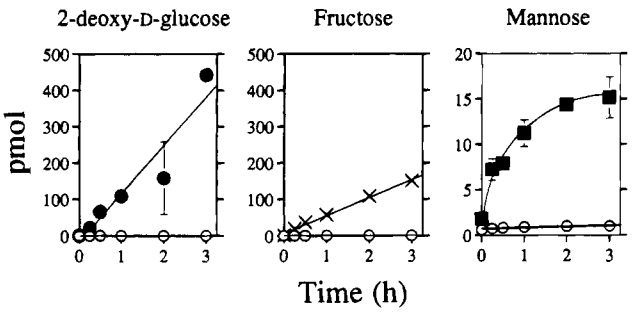

FIGURE 1: Transport of radiolabeled sugars in *Xenopus* oocytes injected with D2 RNA. Injected oocytes were incubated with 50 μM [^{3}H]2-DOG (●), 50 μM [^{14}C]D-fructose (×), or 50 μM [^{3}H]-D-mannose (■). For each time point, uptake into three oocytes was measured and reported as the mean, with error bars representing the standard deviations from the mean. Open circles (○) represent control uninjected oocytes.

whether the D2 gene encodes a functional transporter and to define its substrate specificity, we microinjected *Xenopus* oocytes with D2 cRNA and assayed these oocytes for transport of various radiolabeled sugars. Figure 1 demonstrates that oocytes expressing the D2 gene transport [^{3}H]2-DOG, [^{14}C]D-fructose, and [^{3}H]D-mannose, with a decreasing order of efficiency. Hence, D2 transports the same hexoses as the previously characterized (Langford et al., 1994) Pro-1 transporters. The linear uptake of 2-DOG and fructose over an extended period of time is presumably due to the phosphorylation of these sugars by hexokinase, leading to irreversible trapping of the radiolabeled substrates within the oocyte. Since the uptake of mannose levels off after about 2 h, it is possible that this substrate is not phosphorylated efficiently by the oocyte hexokinase. However, in general, these uptake assays measure both transport and metabolism. Nonetheless, this fact reinforces the conclusion that D2 transports these three sugars, as they must first be translocated across the oocyte membrane before they can be phosphorylated and trapped within the cell.

To further characterize the substrate specificty of the D2 transporter, we performed transport assays on control oocytes and oocytes injected with D2 RNA utilizing various other radiolabeled sugars or nucleosides (Figure 2A). The results show that [^{3}H]2-DOG and [^{3}H]D-glucose are the only compounds, among those tested in this experiment, whose transport is strongly stimulated in D2-injected oocytes. The stereospecificity of the D2 transporter is confirmed by its inability to transport [^{3}H]L-glucose. A low level of stimulation is observed for transport of adenosine and guanosine, but these nucleosides are clearly not good substrates for the D2 transporter, since their uptake is stimulated by less than 3-fold over background compared to the ~50-fold stimulation of 2-DOG uptake.

In a complementary set of experiments, we analyzed the ability of various compounds including sugars, nucleosides, and known inhibitors of other transporters to inhibit the transport of 50 μM [^{3}H]2-DOG by the D2 transporter (Figure 2B). L-Glucose did not inhibit transport at 10 mM but did achieve an approximately 30% inhibition at 250 mM, possibly due to osmotic effects of high sugar concentration on the oocyte. D-Glucose and D-fructose partially inhibited transport at very high concentrations (250−300 mM). Although initially surprising, this result is in accord with our subsequent observation that the D2 transporter has a very high K_m for 2-DOG (Figure 3) and for D-glucose (data not shown) and hence requires high concentrations of these substrates to inhibit transport. Although mannose is a relatively poor substrate for D2 (Figure 1), it is an effective inhibitor at 10 mM concentration, as is D-glucosamine which is not a substrate for the transporter; these sugars presumably have nonproductive modes of binding to the transporter. Furthermore, the observation that the K_m for transport of [^{3}H]-D-mannose by D2-injected oocytes is ~250 mM (data not shown) whereas the K_m for [^{3}H]2-DOG is ~150 mM (Figure 3) demonstrates that mannose is not a high-affinity substrate for the D2 transporter, despite its ability to effectively inhibit this transporter. Mannose is also a good inhibitor but a poor substrate for the high-affinity Pro-1 glucose transporter from *L. enriettii* (Langford et al., 1994).

In addition to the compounds shown in Figure 2B, we have tested the following compounds and have determined that none of them significantly inhibits transport of 50 μM [^{3}H]2-DOG: xylose (10 mM), sucrose (10 mM), maltose (10 mM), lactose (10 mM), raffinose (10 mM), sorbitol (10 mM), mannitol (10 mM), cellobiose (10 mM), melibiose (10 mM), inosine (1 mM), uridine (250 μM), cytidine (250 μM), thymidine (250 μM), and folic acid (250 μM). The observation that none of the other sugars or nucleosides studied here significantly inhibits transport of 2-DOG at the concentrations employed eliminates the possibility that any of these compounds are high-affinity substrates for D2 and further supports the conclusion that D2 is a bona fide glucose transporter, even though its K_m for this substrate is quite high. It is notable that dehydroascorbic acid, which is a substrate for at least some mammalian glucose transporters (Vera et al., 1993), is not an inhibitor of 2-DOG transport in D2-expressing oocytes. Some of the sugars, e.g. fructose and *N*-acetylglucosamine, inhibit transport of 2-DOG in parasites at concentrations that do not inhibit the D2 transporter expressed on oocytes. These results probably reflect differences between the major glucose transporter expressed in promastigotes, Pro-1, and the D2 transporter.

We have also tested several pharmacological reagents that are potent inhibitors of mammalian facilitative glucose transporters for their ability to inhibit transport of 2-DOG by the D2 protein. Cytochalasin B and phloretin partially inhibit transport of the glucose analog in both promastigotes and D2-expressing oocytes, although they require a considerably higher concentration than that required to block the mammalian proteins (Baldwin, 1992). In contrast, forskolin does not affect glucose transport activity in either the intact parasites or the oocytes, even though it is a very effective inhibitor in mammalian cells. Phloridzin, an inhibitor of mammalian Na^+-dependent glucose transporters (Hediger et al., 1987), inhibits D2-mediated transport poorly if at all. Other potential inhibitors of active transport processes are ouabain, which blocks the sodium−potassium ATPase, thus eliminating the plasma membrane Na^+ gradient utilized by many active symporters, DCCD, which inhibits the mitochondrial F_1/F_0 ATPase and hence oxidative phosphorylation, and FCCP, which is a proton ionophore that collapses H^+ gradients and should inhibit proton symporters. Only FCCP has an effect on uptake of 2-DOG in promastigotes or D2-expressing oocytes. However, it is unclear whether this result is the direct effect of inhibition of the D2 transporter or the indirect effect of inhibition of oxidative phosphorylation, leading to a reduction of 2-DOG phosphorylation and a decrease in the amount of substrate that can be trapped inside

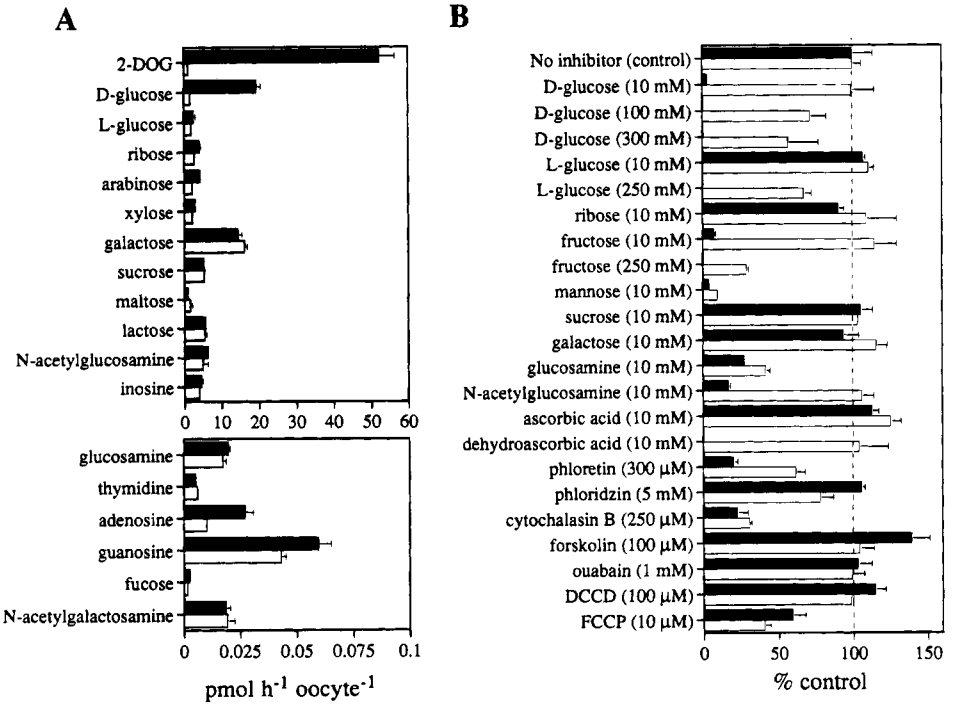

FIGURE 2: Substrate and inhibitor specificity of the D2 transporter. (A) Transport of various radiolabeled compounds by uninjected oocytes (open bars) and by oocytes injected with D2 RNA (solid bars). Error bars represent standard deviations from the mean for at least four oocytes. For all compounds in the top panel, the assays were performed in the presence of 50 μM each labeled compound. For those compounds in the bottom panel, no unlabeled reagent was added, and concentrations of substrates were as follows: glucosamine, 0.050 μM; thymidine, 0.024 μM; adenosine, 0.058 μM; guanosine, 0.18 μM; fucose, 0.023 μM; and *N*-acetylgalactosamine, 0.080 μM. Assays were performed for 1 h. (B) Inhibition of transport of [^{3}H]2-DOG in promastigotes of *L. donovani* (solid bars) and in oocytes (open bars) injected with D2 RNA. For each measurement, the concentration of [^{3}H]2-DOG was 50 μM, and the concentration of inhibitor was as indicated in the figure. Assays in promastigotes were performed for 1 min and those in oocytes for 1 h. Each bar represents the mean of three independent measurements, and error bars indicate standard deviations. The dashed line indicates the average value obtained in the absence of any inhibitor. The absence of solid bars for the following inhibitors indicates that these compounds were tested in oocytes but not in parasites: D-glucose (100 mM), D-glucose (300 mM), L-glucose (250 mM), fructose (250 mM), and dehydroascorbic acid (10 mM).

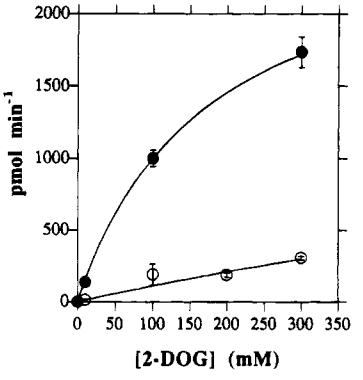

FIGURE 3: Substrate saturation curve for 2-DOG for oocytes injected with D2 RNA. Uninjected oocytes (○) or oocytes injected with D2 RNA (●) were incubated for 10 min with the indicated concentrations of [^{3}H]2-DOG. Each point represents the mean of uptake of ligand (pmol min^{-1}) for three oocytes, with error bars representing standard deviations. The curve is a least squares fit to the Michaelis–Menten equation which produces an estimated K_m of 150 mM for 2-DOG.

the cell as 2-deoxy-D-glucose 6-phosphate. Furthermore, voltage clamping (Langford et al., 1994) of oocytes injected with D2 RNA failed to reveal any transmembrane currents upon addition of 10 mM 2-DOG. Hence, we do not have any evidence for active transport by the D2 protein.

Kinetic Properties of D2 Transporter Expressed in Xenopus Oocytes. Functional distinctions between glucose transporters are often reflected in their kinetic properties. Thus, expression of human GLUT1 and GLUT3 in *Xenopus* oocytes has revealed relatively low K_ms (~17 and ~10 mM, respectively) for the nonmetabolizable glucose analog 3-*O*-methylglucose and a relatively higher K_m (~42 mM) for GLUT2 (Gould et al., 1991), and it has been suggested (Thorens et al., 1990) that these kinetic differences can be attributed to the distinct physiological functions of each transporter subtype. We have used a substrate saturation curve (Figure 3) to estimate a K_m of ~150 mM for 2-DOG for the D2 transporter expressed in oocytes. Hence, the D2 transporter has a much higher K_m for 2-DOG than the two isoforms of the Pro-1 glucose transporter, which have K_ms in the submillimolar range (Langford et al., 1994). Nonetheless, the substrates recognized by these two types of transporter appear to be identical, at least among the ligands we have tested.

Immunolocalization of the D2 Transporter in L. donovani Promastigotes. Another potential distinction between glucose transporter subtypes could involve their subcellular localizations. Thus, human GLUT1 resides primarily in the plasma membrane, GLUT4 in intracellular vesicles (Slot et al., 1991), and GLUT7 in the endoplasmic reticulum (Waddell et al., 1992), and the two different isoforms of the *Leishmania* Pro-1 transporter are differentially sorted to the plasma membrane and the flagellar membrane (Piper et al., 1995). To assess the location of the D2 transporter, we have prepared an affinity-purified antibody, called D2C, directed against the COOH terminal hydrophilic tail. On immunoblots of promastigote lysates (Figure 4), this antibody reacts with a major band of ~60 000 and several minor bands

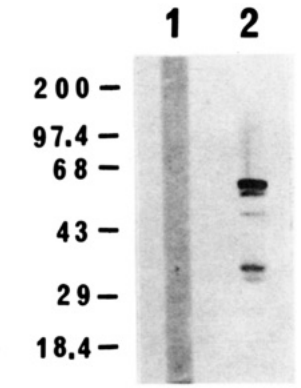

FIGURE 4: Immunoblots of extracts from *L. donovani* promastigotes. Extracts from wild type DI700 promastigotes were separated on a 10% Laemmli gel, electroblotted onto nitrocellulose, and probed with preimmune serum (lane 1) or with affinity-purified D2C antibody (lane 2). The numbers at the left indicate protein molecular weight markers in kilodaltons.

which may be proteolytic products of the native D2 protein (predicted molecular weight, 67 400). Preimmune serum at the same dilution does not detect any bands.

Initial immunolocalization studies using wild type promastigotes and the D2C antibody did not produce strong enough signals to clearly define the distribution of the D2 transporter. Consequently, it was necessary to overexpress the D2 protein by stably transfecting parasites with the multicopy extrachromosomal *Leishmania* expression vector pALT-Neo (Laban et al., 1990) containing the D2 coding sequence inserted into the polylinker cloning site. Immunoblots of this cell line revealed that these parasites overexpressed the D2 protein by an estimated 3−10-fold compared to the wild type parasites. Confocal immunofluorescence images (Figure 5) of this cell line incubated with the D2C antibody reveal staining on the plasma membrane surrounding the cell body and in some cells also in a discrete perinuclear dot. However, the flagellar membrane was not stained. Control incubations with preimmune serum or with D2C antibody blocked by preincubation with the D2-GST fusion protein showed only low levels of background staining over the cell body, while preincubation of the D2C antibody with native GST did not abolish the plasma membrane staining (data not shown).

To define the distribution of the D2 transporter more precisely, we also performed immunoelectron microscopy on frozen thin sections of the overexpressing cell line. Parasites stained with the D2C antibody exhibited gold particles primarily over the plasma membrane (Figure 6A) but also in the region of membrane stacks which may represent the Golgi complex (Figure 6A,C). No gold particles were detected over the flagellum (Figure 6A,B). Control frozen thin sections incubated with preimmune serum (Figure 6D) did not show significant decoration with gold particles. Hence, the results of both immunofluorescence and immunoelectron microscopy indicate that the D2 protein is located on the plasma membrane over the surface of the cell and possibly on internal membranes of the biosynthetic pathway such as the Golgi apparatus but is largely excluded from the flagellar membrane.

DISCUSSION

Expression of the *L. donovani* D2 gene in *Xenopus* oocytes confirms that the D2 protein is a functional hexose transporter

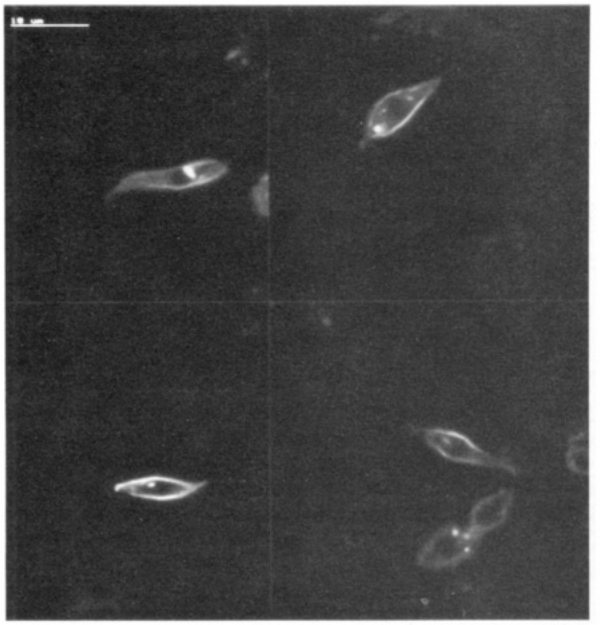

FIGURE 5: Confocal immunofluorescence images of parasites overexpressing the D2 transporter probed with the D2C antibody. Four separate fields showing 0.5 μm sections through each sample. The scale bar in the upper left corner represents 10 μm.

with a substrate specificity identical to that of the Pro-1 transporters from *L. enriettii*. Although the D2 and Pro-1 genes studied were cloned from different *Leishmania* species, both genes exist in both species of parasite (Langford et al., 1992). In each organism, Pro-1 is a multicopy gene family that encodes relatively abundant promastigote-specific mRNAs, and D2 is a single copy gene that encodes a much less abundant promastigote-specific mRNA. Hence, the presence in *Leishmania* parasites of multiple glucose transporters with distinct functional properties has been substantiated.

To our knowledge, the K_m (~150 mM) estimated for the D2 transporter is higher than that reported for any other previously expressed glucose transporter. However, it is noteworthy that another glucose transporter, that from rat liver lysosomal membranes, has an estimated K_m for D-glucose of 90 mM (Mancini et al., 1990); hence, there is at least one other well-characterized glucose transporter with a K_m in the range of the K_m measured for the D2 transporter . Since sandflies, the insect vectors for *Leishmania* parasites, live on plant nectar that is rich in sugars such as sucrose (Schlein, 1986), the parasites are exposed to high concentrations of glucose and fructose following the sandfly sugar meal and the subsequent cleavage of sucrose to glucose and fructose that occurs within the insect gut. Although it is difficult to determine the concentration of sugars within wild sandflies, these insects are fed solutions containing 10% sucrose (300 mM) when they are maintained in the laboratory (Warburg & Schlein, 1986). Consequently, promastigotes are probably exposed, at least transiently, to sugar concentrations in the range of the K_m measured for the D2 transporter. It is possible that the D2 transporter is specialized to function in rapid acquisition of glucose and fructose following the sandfly sugar meal, when the parasite must compete with the sandfly for uptake of this nutrient. The high V_{max} (2692 ± 156 pmol min^{-1} oocyte^{-1}, mean ± standard deviation for $n = 3$) measured for the D2 transporter compared to that for the Pro-1 transporter (34 ± 2 pmol min^{-1} oocyte^{-1}, $n = 3$) would also be consistent with a possible role for D2 in

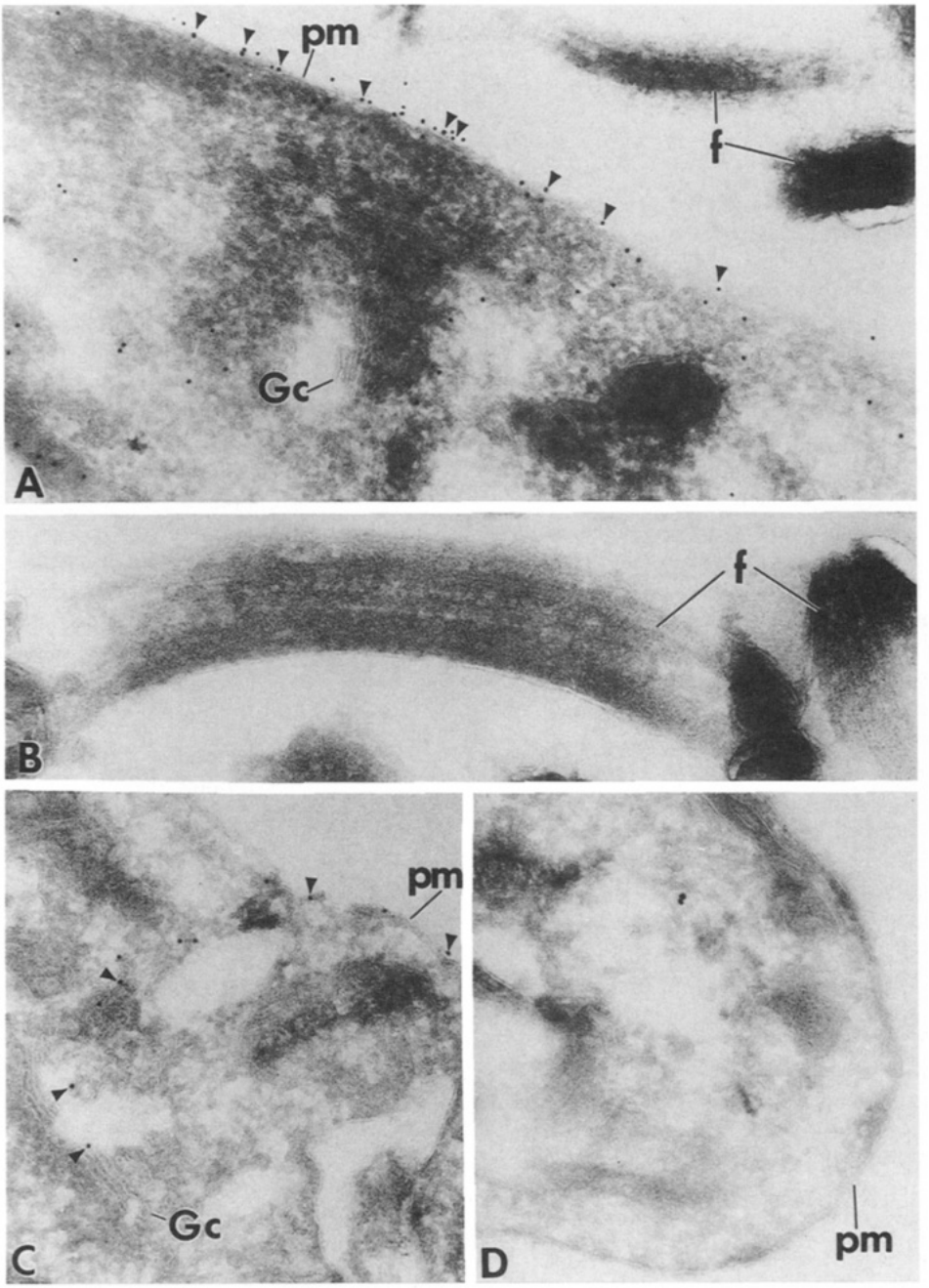

FIGURE 6: Electron micrographs of frozen thin sections from parasites overexpressing the D2 transporter probed with the D2C antibody. (A) Longitudinal section showing staining on the plasma membrane (pm) and in the region of the Golgi complex (Gc) but absence of staining on the flagellum (f), 60000×. Arrowheads point to several gold particles located on the plasma membrane. (B) Section showing absence of staining on an individual flagellum (f), 70000×. (C) Section showing staining in the region of the Golgi complex (Gc), 54000×. Arrowheads point to several gold particles located between the Golgi complex and the plasma membrane. (D) Control section stained with preimmune serum, 54000×. This figure was reproduced at 75% of its original size.

rapid nutrient acquisition. Once glucose has been transported into the parasite, it is rapidly phosphorylated and trapped within the cell, allowing for accumulation of sugar even as the concentration of free glucose in the sandfly gut drops. However, it is also possible that expression of D2 in the heterologous oocyte system might raise the measured K_m somewhat above the true K_m in the intact parasites, due to some unkown differences in protein modification or physiological environment. Since D2 is a minor glucose transporter in the promastigotes, it is not possible to directly measure its kinetic parameters within the parasite.

The very high K_m measured for 2-DOG raises the possibility that the D2 transporter might recognize some other unknown substrate with higher affinity and transport glucose, fructose, and mannose much less effectively than this alternate substrate. We have tested a large number of other potential substrates and found no evidence that any of these compounds are significant substrates for the D2 protein. In particular, the sugars maltose, sucrose, raffinose, and melibiose have been detected in the midguts of sandflies (Young et al., 1980) and hence might be candidate substrates for the D2 transporter. However, the failure of these sugars to inhibit transport of [^{3}H]2-DOG by the D2 transporter confirms that they are not substrates for this carrier. In the absence of positive evidence identifying another substrate, it is possible neither to support nor to rule out the conjecture that D2 might have another higher affinity substrate. Nonetheless, this carrier does transport glucose at concentra-

tions that the parasite is expected to encounter within the sandfly gut, confirming that this protein can function as a glucose transporter under physiological conditions.

Furthermore, the observation that 10 mM D-glucose greatly inhibits transport of 50 μM [^{3}H]2-DOG in parasites (Figure 2B) does not rule out a role for D2 as a glucose transporter. The major glucose transporters in *Leishmania* parasites are members of the Pro-1 family, which are both expressed more abundantly than D2 (Langford et al., 1992) and which have a much lower K_m for 2-DOG. Hence, the great majority of glucose transport by the parasites exposed to 50 μM substrate will be accomplished by these transporters and will be inhibited by unlabeled 10 mM D-glucose. The component of glucose transport mediated by the D2 transporter should be greater at higher glucose concentrations than it is at 50 μM substrate, since the Pro-1 transporters will attain their maximal velocity at moderate glucose concentrations while the velocity of the D2 transporter will continue to increase as higher substrate concentrations are encountered. Although it is not clear a priori why the parasite should express two glucose transporters with very different K_ms in the same life cycle stage, it is possible that future studies involving targeted gene replacements (Cruz et al., 1991) of the D2 gene might reveal an altered phenotype which could elucidate this question.

The observation that high concentrations of D-glucose and L-glucose inhibit transport of 50 μM 2-DOG to similar extents (Figure 2B) might at first appear to contradict the conclusion that D2 functions as a glucose transporter. However, this result is probably the consequence of the high substrate concentrations required to inhibit a high-K_m transporter; the 250−300 mM sugar solutions employed may partially inhibit transport in the oocytes in a nonspecific manner. The direct proof that D2 is stereospecific and transports D-glucose but not L-glucose emerges from radiolabeled transport assays (Figure 2A) which show that the D-isomer but not the L-isomer is transported by D2-injected oocytes.

A region of high sequence identity between D2 and Pro-1, beginning in putative transmembrane helix 11 and extending into putative transmembrane helix 12, was previously noted (Langford et al., 1992). Since the COOH terminal region of GLUT1 interacts with substrate analogs in affinity-labeling experiments (Walmsley, 1988), it is possible that the analagous highly conserved segment of D2 and Pro-1 is involved in substrate binding. This speculation is supported by the current observation that D2 and Pro-1 do share the same substrates and hence may have related substrate binding sites.

Immunolocalization studies indicate that D2 is a plasma membrane transporter. This result underscores a similarity with the plasma membrane isoform of Pro-1 and a difference with the flagellar membrane isoform of that transporter. Furthermore, these results preclude the possibility that D2 is located in the membrane of the glycosomes, intracellular organelles that are distributed throughout the cytoplasm and contain the glycolytic enzymes in *Leishmania* and related kinetoplastid protozoa (Hart & Opperdoes, 1984), and which might require organelle-specific glucose transporters. It is possible that additional glucose transporters remain to be identified in these parasites.

One potential concern involves the use of overexpressing cell lines in the immunolocalization studies. In principle, such overexpression could lead to alterations in the subcellular distribution of D2 proteins. However, the observation that overexpression is less than 10-fold makes a major redistribution of transporters seem unlikely. It is possible that the relative abundance of D2 in the perinuclear dot, which might represent the Golgi complex or other components of the membrane biosynthetic apparatus, is more pronounced in overexpressers than in wild type parasites due to accumulation of transporters along the route of membrane assembly. However, the plasma membrane residence we observe for D2 is typical for many transporters and is not the result expected for mislocalization artifacts.

It is also intriguing that D2 is largely excluded from the flagellar membrane like the major Pro-1 isoform but unlike the minor Pro-1 isoform. This result further reinforces the functional differences between the flagellar membrane and the plasma membrane in these parasites. This differential distribution of various glucose transporter isoforms between these two membranes in *Leishmania* parasites is quite different from the example of human GLUT5 fructose transporters, which are evenly distributed between the plasma and flagellar membranes of spermatozoa (Burant et al., 1992).

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