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Functional Expression of a *myo*-Inositol/H^+ Symporter from *Leishmania donovani*

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The vast majority of surface molecules in such kinetoplastid protozoa as members of the genus *Leishmania* contain inositol and are either glycosyl inositol phospholipids or glycoproteins that are tethered to the external surface of the plasma membrane by glycosylphosphatidylinositol anchors. We have shown that the biosynthetic precursor for these abundant glycolipids, *myo*-inositol, is translocated across the parasite plasma membrane by a specific transporter that is structurally related to mammalian facilitative glucose transporters. This *myo*-inositol transporter has been expressed and characterized in *Xenopus laevis* oocytes. Two-electrode voltage clamp experiments demonstrate that this protein is a sodium-independent electrogenic symporter that appears to utilize a proton gradient to concentrate *myo*-inositol within the cell. Immunolocalization experiments with a transporter-specific polyclonal antibody reveal the presence of this protein in the parasite plasma membrane.

Leishmania donovani is a parasitic protozoan that spends its entire life cycle within either an insect vector or a mammalian host. Promastigotes live within the midgut or proboscis of the sandfly and are injected into the vertebrate host when the sandfly takes a blood meal. Following deposition within the skin of the host, the flagellated promastigotes are engulfed by macrophages in which they transform into nonflagellated amastigotes that are specialized for survival within the phagolysosomal vesicles. Hence, these parasites are dependent upon their hosts for provision of nutrients, which are then utilized either catabolically for generation of energy or metabolic degradation products or anabolically for biosynthesis of parasite macromolecules.

One striking distinction between the mammalian host and the parasite involves the composition of the plasma membrane. The great majority of surface molecules in *Leishmania* cells are either inositol-containing phospholipids or glycoproteins that are anchored into the plasma membrane by glycosylphosphatidylinositol anchors (24). Thus, the major glycolipid (39) in promastigotes, lipophosphoglycan, is an inositol phospholipid that undergoes structural modifications during the life cycle and is involved in the invasion of macrophages and in attachment of the parasite to the epithelium of the insect midgut. The major glycoprotein of the cell surface, gp63, has been implicated in the invasion of macrophages (8, 29) and is attached to the parasite surface by a glycosylphosphatidylinositol anchor (5). Finally, there is a diverse family of glycosylinositol phospholipids that form a glycocalyx covering the surface of both promastigotes and amastigotes but whose specific functions remain to be determined (24). The biosynthetic pathways of glycosylphosphatidylinositol anchors (24) and lipophosphoglycan (39) have been determined, and they both require phosphotidyl inositol as a building block. Hence, the polyalcohol *myo*-inositol, itself a precursor for phosphatidylinositol biosynthesis, may be required for lipid biosynthesis and could be provided by either de novo biosynthesis or salvage from the extracellular medium or by both.

In a previous study (19), we cloned and sequenced a gene from *L. donovani* which encodes a protein with significant sequence similarity to those of members of the facilitative glucose transporter superfamily (13) and which is called D1. Comparison of its sequence with other sequences revealed a marked similarity between the D1 protein and two inositol transporters that had been cloned from the yeast *Saccharomyces cerevisiae* (25). This sequence similarity prompted us to express the D1 gene in *Xenopus laevis* oocytes and to assay for the acquisition of *myo*-inositol transport activity. Characterization of D1-expressing oocytes confirms that this protein is a bona fide *myo*-inositol transporter and reveals that it is an electrogenic symporter that couples proton and inositol transport to allow active concentration of the substrate. We have also investigated transport of *myo*-inositol by promastigotes of *L. donovani* and have measured a similar transport activity that apparently represents the operation of the D1 transporter in intact parasites. These results indicate that *Leishmania* parasites can salvage this important building block from the extracellular medium via a specific plasma membrane carrier.

MATERIALS AND METHODS

Growth of cells and preparation of oocytes. Promastigotes of the DI700 clone of *L. donovani* were grown in DME-L medium (15) supplemented with 0.25% hemin and 100 μM xanthine at 27°C to a density of 1×10^7 to 2×10^7 cells ml^{-1}, pelleted by centrifugation, washed once in phosphate-buffered saline (PBS) (pH 7.4), and resuspended in PBS at a density of 5×10^8 cells ml^{-1} for transport assays. *Xenopus* oocytes were prepared and microinjected with D1 cRNA as described previously (21).

Plasmid constructs and transfections. For expression of the D1 gene in oocytes, the D1.SH plasmid (19) containing the coding region of the D1 protein was modified by PCR (30) to remove most of the 5′ untranslated region and to introduce a *Bam*HI restriction site upstream from the initiation codon. The region of the plasmid that had been amplified by PCR was recloned into the D1.SH plasmid and sequenced to ensure that no alterations had occurred in the protein coding region. The *Bam*HI-*Hin*dIII insert of this plasmid was subcloned into the *Bgl*II-*Hin*dIII sites of the *Xenopus* expression vector pL2-5 (2), capped cRNA was prepared from this vector as described previously (21), and 50 nl containing ~20 ng of RNA was injected into each oocyte. For overexpression of the D1 gene in *L. donovani* promastigotes, the *Sma*I-*Hin*dIII insert of the D1.SH

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plasmid was subcloned into a vector that placed *Bam*HI sites on both sides of the insert and the *Bam*HI insert of this vector was then subcloned into the *Bam*HI site of the pX expression vector (22) to produce the D1pX plasmid. The D1pX plasmid was transfected into *L. donovani* promastigotes as reported previously (28), and the transfectants were selected in liquid medium containing 200 μg of G418 per ml.

Transport assays. For transport assays of promastigotes, 100 μl of cells at 5×10^8 cells ml^{-1} was mixed with 100 μl of radiolabeled ligand at the appropriate concentration (typically 10 μM to 10 mM, with a specific activity of 1×10^6 to 5×10^6 μCi $mmol^{-1}$) and layered over 100 μl of dibutyl phthalate, and this mixture was incubated for the appropriate length of time and then spun in an Eppendorf microcentrifuge for 30 s. The pelleted samples were processed as described previously (21). For measurements of transport velocities for substrate saturation curves, incubations were performed for 0, 1, 3, 7, and 10 min. For the measurements at 0 min, the cells were preincubated in 1% formaldehyde to ensure immediate stopping of the carrier-mediated transport reaction. Control experiments demonstrated that cells preincubated in 1% formaldehyde did not accumulate any radiolabeled material beyond the background level present at 0 min. For each datum point, the picomoles of labeled substrate transported were calculated and plotted as a function of incubation time. These data were fit to a straight line by a linear regression analysis with Kaleidagraph software (Synergy Software, Reading, Pa.), and a velocity of transport at the given substrate concentration was calculated from the slope of this line. All linear regression plots yielded r values of ≥0.96. For measurements of inhibition of uptake by various inhibitors, cells were preincubated with or without inhibitors for 10 min and then with 50 μM radiolabeled inositol for 10 min before being pelleted through oil.

For transport measurements with oocytes, the oocytes were incubated with radiolabeled substrate at the appropriate concentration for 30 min and then rinsed and prepared for liquid scintillation counting as described previously (21). Control experiments demonstrated that the uptake of substrate was linear over this 30-min incubation.

Two-electrode voltage clamp experiments. Two-electrode voltage clamp recordings of oocyte membrane currents were made essentially as described previously (17). Recordings were made with a Geneclamp 500 amplifier (Axon Instruments, Foster City, Calif.) interfaced via a MacLab A/D converter (AD Instruments, Castle Hill, New South Wales, Australia) to a Macintosh Quadra. Oocytes were voltage clamped at −90 mV and continuously superfused with ND96 buffer alone or buffer containing substrate at the concentration indicated. Currents (I) induced by substrate application were fitted by least squares to the equation $I = I_{max}$ ([substrate]/[substrate] + K_m). Values for K_m are expressed as the means ± standard errors of the means from fits to individual oocytes, and curve fits to normalized mean data for all oocytes tested are also given (see Fig. 6A).

Preparation and use of affinity-purified antibody. For preparation of the D1-COOH antibody, the segment of the D1.SH clone encoding the 136-amino-acid hydrophilic COOH-terminal domain (the D1C peptide) was amplified by PCR and subcloned into the *Bam*HI site of pGEX-2T (34) for generation of a D1-glutathione *S*-transferase fusion protein. This fusion protein was prepared and injected into rabbits, and antisera were collected and affinity purified as described previously (28), except that the affinity column contained the 136-amino-acid D1C peptide that had been cleaved from the fusion protein with thrombin and purified by chromatography over a glutathione affinity column (34). This affinity-purified antibody was typically used at a 1:1,000 to 1:2,000 dilution.

Protein blots were performed as described previously (28) with an enhanced chemiluminescence kit (Amersham Corp., Arlington Heights, Ill.). Protein samples were incubated at 65°C for 5 min in Laemmli sample buffer (31) at the time of initial cell lysis and immediately before they were applied to sodium dodecyl sulfate–10% polyacrylamide gels. Incubations of lysates at 95°C resulted in massive aggregation of the D1 protein and trapping at the top of the gel. Incubations of antibody with blocking peptides were performed with the D1C peptide or with the unrelated P3 peptide (HPWDEERDGKKVVAPAIGKKELSEESIGNRAE) that represents the COOH-terminal hydrophilic domain of the *Leishmania enriettii* Pro-1 glucose transporter (6). For these blocking reactions, 2.5 μl of affinity-purified D1-COOH antibody was diluted into 100 μl of TBST (20 mM Tris-HCl [pH 7.6], 150 mM NaCl, 1% Tween 20) containing 25 μg of peptide, and this mixture was incubated for 1 h at room temperature, diluted into 5 ml of TBST plus 5% powdered milk, and incubated with the protein blot.

For confocal immunofluorescence microscopy, parasites were fixed with methanol, stained with a 1:1,000 dilution of D1-COOH antibody, incubated with goat anti-rabbit immunoglobulin G conjugated to fluorescein isothiocyanate, and examined with a Leica confocal laser scanning microscope (Leica Lasertechnik GmbH, Heidelberg, Germany) with a Leitz 63× oil immersion lens as described previously (28). Plasma membrane staining was not observed in parasites that had been stained with preimmune serum or with D1-COOH antibody that had been preincubated with the D1C peptide. Immunoelectron microscopy was performed on frozen thin sections of the D1pX.1 line of parasites as described previously (28). Sections were stained with goat anti-rabbit immunoglobulin G conjugated to 15-nm-diameter gold particles.

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ITR1  MGIHIPYLTSKTSQSNVGDAVGNADSVEFNSEHDSPSKRGKIHIESHEIQRAPASDDEDR   60

D1                                MRASVMLCAALGGFLFGYDTGVINAALFQMKDHFGF   36
ITR1  IQIKPVNDEDDTSVMITFNQSLSPFIITLTFVASISGFMFGYDTGYISSALISIGTDLDH  120

D1    SEHSW-QYALIVAIAIAGAFVGAFISGFISAAFGRRPCIAVADALFVIGSVLMGAAPNVE   95
ITR1  KVLTYGEKEIVTAATSLGALITSIFAGTAADIFGRKRCLMGSNLMFVIGAILQVSAHTFW  180

D1    VVLVSRVIVGLAIGISSATIPVYLAEVTSPKHRGATIVLNNLFLTGGQFVAAGFTAIMVV  155
ITR1  QMAVGRLIMGFGVGIGSLIAPLFISEIAPKMIRGRLTVINSLWLTGGQLVAYGCGAGLNY  240

D1    FTSKNIGWRVAIGIGALPAVVQAFCLLFFLPESPRWLLSKGHADRAKAVADK-FE-----  209
ITR1  VNN---GWRILVGLSLIPTAVQFTC-LCFLPDTPRYYVMKGDLARATEVLKRSYTDTSEE  296

D1    ---------VDLCEFQEGDELP-SVRIDYRPLMARDMRFR-VVLSSGLQIIQQFSGINTI  258
ITR1  IIERKVEELVTLNQSIPGKNVPEKVWNTIKELHTVPSNLRALIIGCGLQAIQQFTGWNSL  356

D1    MYYSSVILYDAGFRDAIMPVVLSIPLAFMNALFTAVAIFTVDRFGRRRMLLISVFGCLVL  318
ITR1  MYFSGTIFETVGFKNSSAVSIIVSGTNF---IFTLVAFFSIDKIGRRTILLIGLPG-MTM  412

D1    LVVIAIIGF-FIGTRISYSVGGGLFLALLAVFLALYAPGIGCIPWVIMGEIFPTHLRTSA  377
ITR1  ALVVCSIAFHFLGIKFDGAVAVVVSSGFSSWGIVIIVFIIVFAAFYALGIGTVPWQQSEL  472

D1    ASVATMANWGANVLVSQVFPILMGAIGVGGTFTIISGLMALGCIFVYFFAVETKGLTLEQ  437
ITR1  FPQNVRGIGTSYATATNWAGSLVIASTFLTMLQNITPAGTFAFFAGLSCLSTIFCYFCYP  532

D1    IDNMFRKRAGLPPRFHEEGESGESGAGYREDGDLGRLATEDVCDLSSLGNRVVSFAKAED  497
ITR1  ELSGLELEEVQTILKDGFNIKASKALAKKRKQQVARVHELKYEPTQEIIEDI          584

D1    AFTEVAMPDRHAVSNKFEERATSSSSDPQSLENQDEVRQAAIKAAPHEPK            547
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FIG. 1. Alignment of *L. donovani* D1 protein sequence (upper lines) with *S. cerevisiae* ITR1 *myo*-inositol transporter sequence (lower lines). Vertical lines indicate identical amino acids. Overlining and underlining with numerals 1 to 12 designate the putative transmembrane domains for each transporter (19, 25). Hyphens indicate gaps introduced to maximize alignment. The alignment was generated with the FASTA algorithm (27).

RESULTS

Similarity of D1 protein to yeast *myo*-inositol transporter. The D1 gene from *L. donovani* was originally detected by a PCR-based search for genes that were related in sequence to that of the *Leishmania* Pro-1 glucose transporter (19). The deduced amino acid sequence of the D1 protein revealed that it was significantly related to about 30 known members of the facilitated glucose transporter superfamily, with the highest level of similarity (FASTA [27] similarity search) being with the arabinose transporter from *Escherichia coli*; however, the biochemical function of the D1 polypeptide has not been defined. A more recent search with the BLAST algorithm (1) revealed significant similarity to two yeast *myo*-inositol transporters (25) whose sequences had subsequently been entered into the GenBank database. An optimized alignment of the D1 protein and yeast ITR1 sequences is shown in Fig. 1, and it revealed several clusters of pronounced identity, especially within putative transmembrane segments 1, 3, 5, and 7 of the D1 sequence. The similarity of D1 and ITR1 encouraged us to express the D1 gene in the *Xenopus* oocyte system (9), which we had previously used to characterize the Pro-1 glucose transporter (21), to determine whether the D1 protein functions as an inositol transporter.

Functional expression of the D1 *myo*-inositol transporter in *Xenopus* oocytes. Proof that the D1 protein is a transporter for *myo*-inositol was obtained by injecting *Xenopus* oocytes with cRNA synthesized from the cloned D1 gene and assaying both injected and uninjected control oocytes for transport of

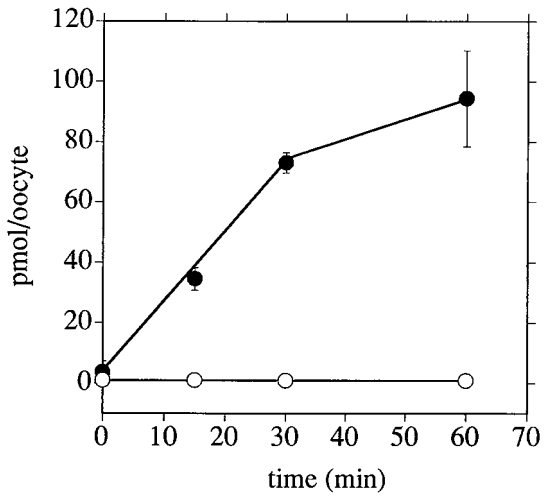

FIG. 2. Time course for uptake of 50 μM [^{3}H]*myo*-inositol by oocytes injected with D1 RNA (solid circles) or by control uninjected oocytes (open circles). D1-expressing oocytes were assayed 3 days after injection with cRNA. Each datum point represents the average and standard deviation (error bars) of measurements from three oocytes. In a similar experiment, control oocytes injected with 50 nl of H_2O took up less [^{3}H]*myo*-inositol than control uninjected oocytes (time points assayed were 0, 30, and 60 min), confirming that uptake of labeled substrate in D1-injected oocytes was not due to the physical manipulations associated with microinjection.

[^{3}H]*myo*-inositol. The results illustrated in Fig. 2 demonstrate that D1-injected oocytes transport labeled *myo*-inositol for at least 1 h and accumulate this substrate to about a 100-fold level above that of uninjected oocytes at the 1-h time point. Similar experiments (data not shown) performed with radiolabeled 2-deoxy-D-glucose, D-arabinose, D-galactose, D-fructose, D-ribose, D-xylose, sucrose, adenosine, inosine, guanosine, thymidine, and folate failed to detect significant transport above the levels observed in control uninjected oocytes, confirming the specificity of the transporter. As expected, uptake of labeled *myo*-inositol was specifically inhibited by excess unlabeled *myo*-inositol but not by unlabeled D-glucose (see Fig. 4). To further investigate the properties of the D1 inositol transporter, we measured *myo*-inositol substrate saturation curves (four independent experiments) for D1-expressing oocytes (Fig. 3A). These curves exhibit Michaelis-Menten kinetics, as was expected for saturable carrier-mediated transport, with a K_m of 0.47 ± 0.08 mM and a V_{max} of 13.8 ± 1.8 pmol min^{-1} oocyte^{-1} (means ± standard errors of the means; $n = 4$).

Transport of *myo*-inositol by *L. donovani* promastigotes. The identification of the D1 protein as a *myo*-inositol transporter by using a heterologous expression system predicts that the intact parasites should also display *myo*-inositol transport activity. To test this prediction, we measured uptake by *L. donovani* promastigotes at various concentrations of [^{3}H]*myo*-inositol and at various times from 0 to 10 min and calculated initial transport velocities at each substrate concentration from these linear time courses. A representative substrate saturation curve is shown in Fig. 3B, and the results of four independent experiments produced a K_m of 0.25 ± 0.05 mM and a V_{max} of 55.5 ± 8.8 pmol min^{-1} 5 × 10^7 cells^{-1}. Hence, the parasites transport this substrate, and the carrier exhibits a K_m which is similar to that measured in D1-expressing oocytes. The modest difference between the K_m measured in oocytes and the K_m determined in parasites might result from differences in the physiological environment of the two cell types or differences in posttranslational modifications. The double reciprocal plot constructed from this saturation curve is linear (Fig. 3B, inset),

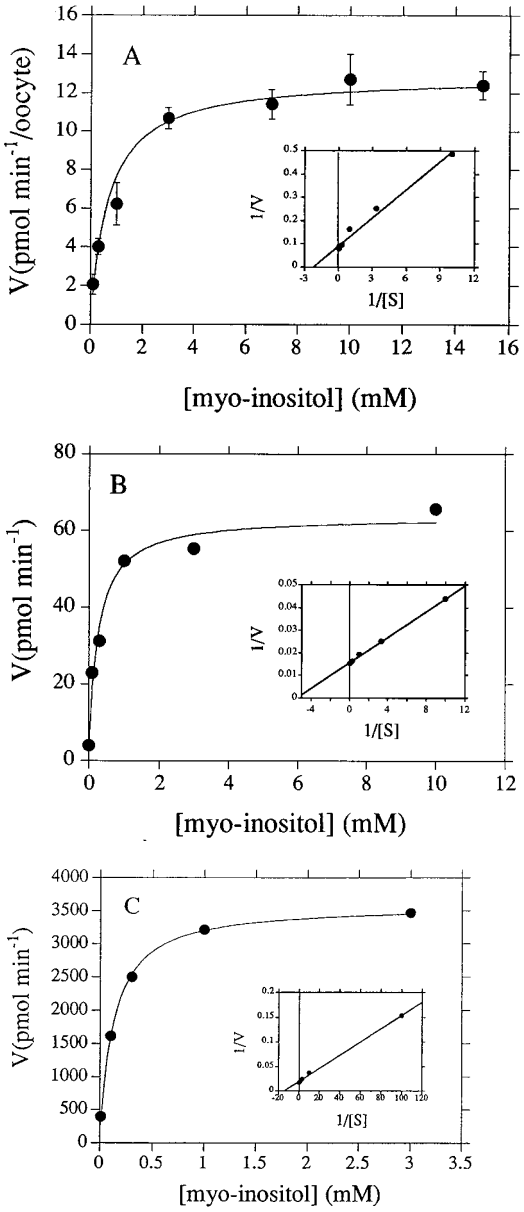

FIG. 3. Uptake of [^{3}H]*myo*-inositol by oocytes injected with D1 RNA (A), by wild type DI700 promastigotes (B), and by the D1pX.1-overexpressing cell line (C). (A) For each datum point, three oocytes were incubated with the radiolabeled inositol for 30 min, and the individual velocities were averaged; error bars indicate standard deviations. (B and C) Each datum point represents a composite of five transport measurements, as described in Materials and Methods, and velocities of *myo*-inositol uptake are reported as picomoles per minute per 5 × 10^7 cells. Curves for velocity (V) versus substrate concentration (S) were fit to the Michaelis-Menten equation ($V = V_{max}S/[K_m + S]$) with the Kaleidagraph program (Synergy Software) and the Levenberg-Marquardt algorithm. The insets display double reciprocal plots of the data in each curve.

consistent with the presence of a single dominant transporter for *myo*-inositol in the parasite membrane.

Overexpression of the D1 transporter in *L. donovani* promastigotes. The preceding results suggested that overexpression of the D1 gene in *L. donovani* would result in increased velocity of *myo*-inositol transport. To test this prediction and further confirm the identity of the D1 transporter, we subcloned the D1 coding region into the pX expression vector (22) to generate the D1pX plasmid, and we stably transfected *L.*

donovani promastigotes with this construct. Selection of transfectants with G418 produced the D1pX.1 cell line. Southern blot analysis of genomic DNA from the D1pX.1 cells compared with that of DNA from wild-type DI700 cells revealed ~200 copies of the D1pX plasmid per cell (data not shown). Northern (RNA) blot analysis of RNA from the D1pX.1 cells revealed pronounced overexpression of multiple RNAs which hybridized to the D1 coding region probe and ranged in size from ~2 to >10 kb (data not shown). Finally, analysis of lysates of D1pX.1 and DI700 cells on protein blots with an affinity-purified antibody directed against the D1 polypeptide (see below) indicated that the overexpressing cell line produced ~100-fold more D1 protein than did the wild-type parasites (data not shown).

A substrate saturation curve for *myo*-inositol transport in D1pX.1 cells (Fig. 3C) revealed a K_m of 0.12 mM and a V_{max} of 3,590 pmol min^{-1} 5×10^7 $cells^{-1}$. Hence, the ratio of 65 for the V_{max} for D1pX.1 cells divided by the V_{max} for DI700 cells is similar to the ~100-fold difference in D1 protein expression and further confirms the identity of the D1 protein as a *myo*-inositol transporter. The approximate parallel between the increase in D1 protein expression and V_{max} for transport further suggests that transport is the rate-limiting step in *myo*-inositol uptake in DI700 cells and that subsequent steps in substrate metabolism are unlikely to significantly affect the kinetic parameters measured for transport.

Inhibition of *myo*-inositol transport in parasites and in D1-expressing oocytes by various compounds. Many members of the glucose transporter superfamily (13) are facilitative transporters that equilibrate substrates across the plasma membrane, but some members, such as the *E. coli* arabinose and xylose transporters (23) and the *Chlorella* (32) and *Arabidopsis* (33) glucose transporters, are active symporters that couple the transport of protons down an electrochemical gradient to drive the concentration of the principal substrate. In addition, a different family of glucose transporters present in mammalian epithelial cells are Na^+-dependent symporters that utilize the plasma membrane Na^+ gradient to concentrate glucose within the cells (40). Such drugs as carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) and dinitrophenol (DNP), which collapse transmembrane proton gradients, NaN_3, which poisons oxidative phosphorylation and thus reduces ATP levels and the processes that rely upon ATP hydrolysis, and oubain, which inhibits eukaryotic sodium-potassium ATPases and destroys transmembrane Na^+ gradients, can be used to examine whether a transport process is active or facilitative. Figure 4 provides a quantitation of *myo*-inositol transport in D1pX.1 cells and in D1-expressing oocytes in the presence and absence of various potential inhibitors. The proton uncouplers FCCP and DNP are quite effective at inhibiting transport, NaN_3 is moderately effective, and oubain has no effect on transport. These results are consistent with the notion of the D1 protein functioning as a proton symporter but require further confirmation from other experiments. Similarly, cytochalasin B and phloretin, which inhibit facilitative glucose transporters (3), are moderately effective inhibitors of the D1 transporter, but phlorizin, an inhibitor of Na^+-dependent glucose transporters, does not affect the D1 transporter. In both D1-expressing oocytes and parasites, two steroisomers of *myo*-inositol, *scyllo*- and *epi*-inositol, partially inhibit transport of the principal substrate; however, we do not know whether these isomers are transported by the D1 carrier or whether they serve as inhibitors rather than substrates. The sugar D-arabinose also partially inhibits transport of *myo*-inositol in oocytes but not in cells, while the sugars D-glucose and D-ribose do not inhibit inositol transport in the oocytes and slightly activate inositol

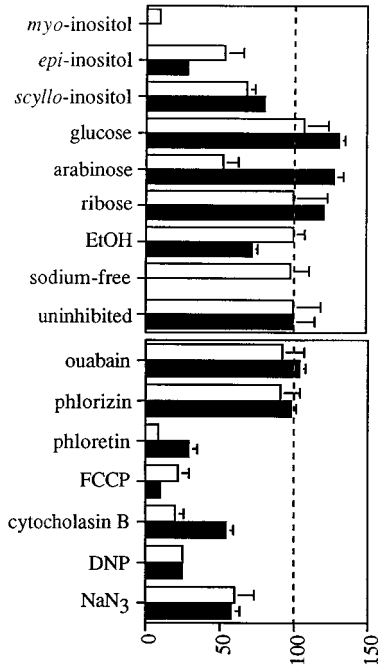

FIG. 4. Inhibition of transport of 50 μM [^{3}H]*myo*-inositol in D1pX.1 cells (solid bars) and in D1-expressing oocytes (open bars) by various compounds. Uptake assays were performed for 10 min on parasites and for 30 min on oocytes. Each bar represents the average and standard error (error bars) of at least three measurements. The concentrations of inhibitors were as follows: unlabeled *myo*-inositol, 10 mM; *scyllo*- and *epi*-inositol, 10 mM; D-glucose, 10 mM; D-arabinose, 10 mM; D-ribose, 10 mM; ethanol (EtOH), 1%; ouabain, 1 mM; phlorizin, 5 mM; phloretin, 300 μM; FCCP, 10 μM; cytochalasin B, 500 μM; DNP, 1.5 mM; and NaN_3, 10 mM. Compounds in the top box were dissolved in H_2O, and uptake levels were normalized to the H_2O control. Compounds in the bottom box were dissolved in 1% ethanol, and uptake levels were normalized to the 1% ethanol control. The dotted line represents the 100% level for uninhibited transport. The transport of labeled *myo*-inositol in the presence of 10 mM unlabeled *myo*-inositol in D1pX.1 cells is not visible in this graph because it was only 0.5% of the uninhibited level. For transport assays in sodium-free medium, Na^+ was replaced with the same concentration of choline. These sodium-free experiments were performed with D1-expressing oocytes but not with parasites.

transport in parasites. We do not understand the quantitative discrepancy between the results from oocytes and cells.

pH dependence of *myo*-inositol transport. If the D1 transporter is a proton symporter, the kinetic parameters for transport should be affected by pH. To test this possibility, we measured substrate saturation curves for D1-expressing oocytes at pH 6.5, 7.5, and 8.5 (Fig. 5). These curves demonstrate that the V_{max} does increase from 11.1 to 21.7 pmol min^{-1} $oocyte^{-1}$ as the pH is dropped from 8.5 to 6.5. Furthermore, the transport of radiolabeled *myo*-inositol is not affected by removal of Na^+ from the oocyte bathing buffer (Fig. 4), indicating that Na^+ is not the counterion for inositol transport. These results further support the identification of D1 as a *myo*-inositol/H^+ symporter.

Demonstration of electrogenic symport by two-electrode voltage clamp experiments with D1-expressing oocytes. A direct way to test for electrogenic symport and to examine the counterion requirement is to measure the transmembrane currents generated during *myo*-inositol transport in D1-expressing oocytes by the two-electrode voltage clamp method (17). Figure 6A shows that addition of *myo*-inositol to D1-expressing oocytes induces transmembrane conductances and that the magnitude of these currents is dependent upon the *myo*-inositol concentration. The K_m calculated from the dose-response curve (Fig. 6B) is 0.22 ± 0.02 mM (n = 3), which is in reason-

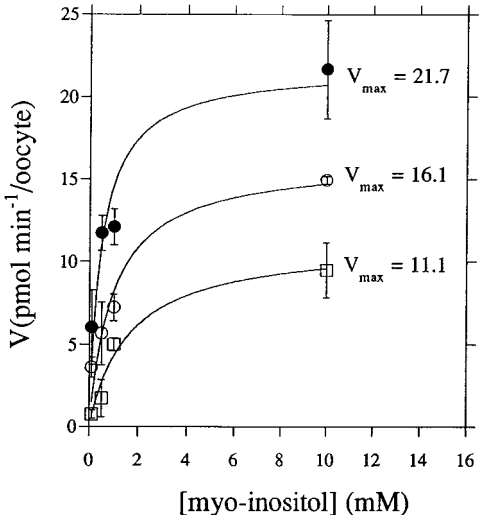

FIG. 5. pH dependence of *myo*-inositol transport in D1-expressing oocytes. Transport of [^{3}H]*myo*-inositol was determined with oocytes incubated in ND96 buffer which had been adjusted to pH 6.5 (●), 7.5 (○), or 8.5 (□). Each datum point represents the average and standard deviation for three oocytes. These data were fit to the Michaelis-Menten equation to determine the V_{max} at each pH. All measurements were made with oocytes derived from the same preparation.

able agreement with the K_m measured by the radiolabeled transport assays of D1-expressing oocytes. Furthermore, these currents are not reduced by replacing Na^+ in the bathing buffer with choline (Fig. 6A), confirming that Na^+ is not the counterion for the D1 transporter, although it is the counterion for many other active transporters. Collectively, these results strongly suggest that the D1 transporter is a *myo*-inositol/H^+ symporter.

Immunolocalization of the D1 transporter in *L. donovani* promastigotes. To assess the localization of the D1 protein in the parasite, we have raised an affinity-purified polyclonal antibody against the COOH-terminal hydrophilic domain of the D1 polypeptide. The specificity of this antiserum (designated D1-COOH) for the D1 protein was examined with protein blots (Fig. 7). Lysates from D1pX.1 cells (Fig. 7, lane 2) which overexpress the D1 protein or from DI700 cells (data not shown) generate a single band with an apparent size of ~50 kDa when they are reacted with the affinity-purified antibody. This band is not present when preimmune serum is used to develop the blot (Fig. 7, lane 1) or when the D1 peptide is used to inhibit the signal (lane 3). Competition with an irrelevant P3 peptide (Fig. 7, lane 4) at the same concentration does not eliminate the 50-kDa band.

The antiserum was employed in both confocal immunofluorescence and immunoelectron microscopy. Wild-type DI700 parasites did not stain detectably with this antiserum, indicating that the D1 protein is not very abundant in the parasite membrane. This result was expected, as the D1 mRNA is present at a relatively low level in both promastigotes and amastigotes (19). Hence, it was necessary to use the D1pX.1 overexpresser line for localization of the D1 polypeptide. Figure 8A presents a confocal immunofluorescence image of two promastigotes stained with the D1-COOH antiserum and reveals strong staining on the parasite plasma membrane and weak punctate staining over the flagellum. Similarly, immunoelectronmicroscopy of frozen thin sections (Fig. 8B and C) from these parasites reveals gold particles over the plasma membrane and also on the flagellar membrane but does not show any significant intracellular staining. Hence, the D1 protein is expressed on the cell surface, as was anticipated from

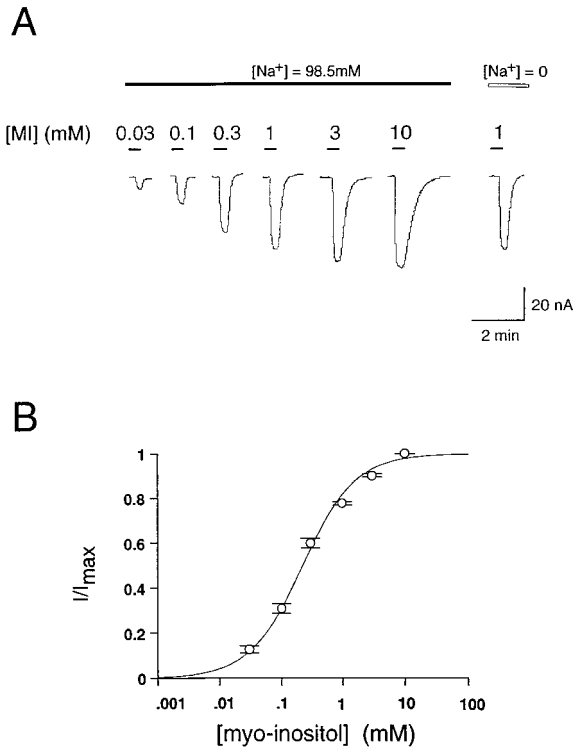

FIG. 6. Measurement of *myo*-inositol-dependent transmembrane conductances in D1-expressing oocytes by the two-electrode voltage clamp method. (A) Oocytes were injected with 20 ng of D1 cRNA and incubated at 17°C for 3 days. Each oocyte was impaled with two electrodes, clamped to a holding potential of −90 mV, and continuously superfused with ND96 buffer ([Na^+] = 98.5 mM) or with choline 96 buffer ([Na^+] = 0). Oocytes were then superfused with buffer containing the indicated concentration of *myo*-inositol [MI] and then washed out with buffer containing no inositol. Traces represent currents recorded during the application and washout of *myo*-inositol. (B) The ratio of the steady state current (I) to the maximum current (I_{max}) in 10 mM *myo*-inositol was plotted against the logarithm of *myo*-inositol concentration. Points represent the means ± standard errors for measurements with three oocytes.

previous characterization of transport activity in wild-type and D1pX.1 cells.

DISCUSSION

Inositol transport in *L. donovani* and other organisms. Inositol has been shown to be present in a wide variety of both prokaryotic and eukaryotic organisms and is most often found as inositol phospholipid components of biological membranes.

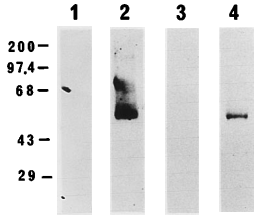

FIG. 7. Protein blots of lysates from D1pX.1 cells probed with the affinity-purified D1-COOH antiserum. Lane 1 was probed with preimmune serum, lane 2 was probed with the D1-COOH antibody, lane 3 was probed with the D1-COOH antibody in the presence of competing D1C peptide, and lane 4 was probed with D1-COOH antibody inhibited by the irrelevant P3 peptide. Numbers at the left indicate the molecular masses in kilodaltons of protein molecular mass markers.

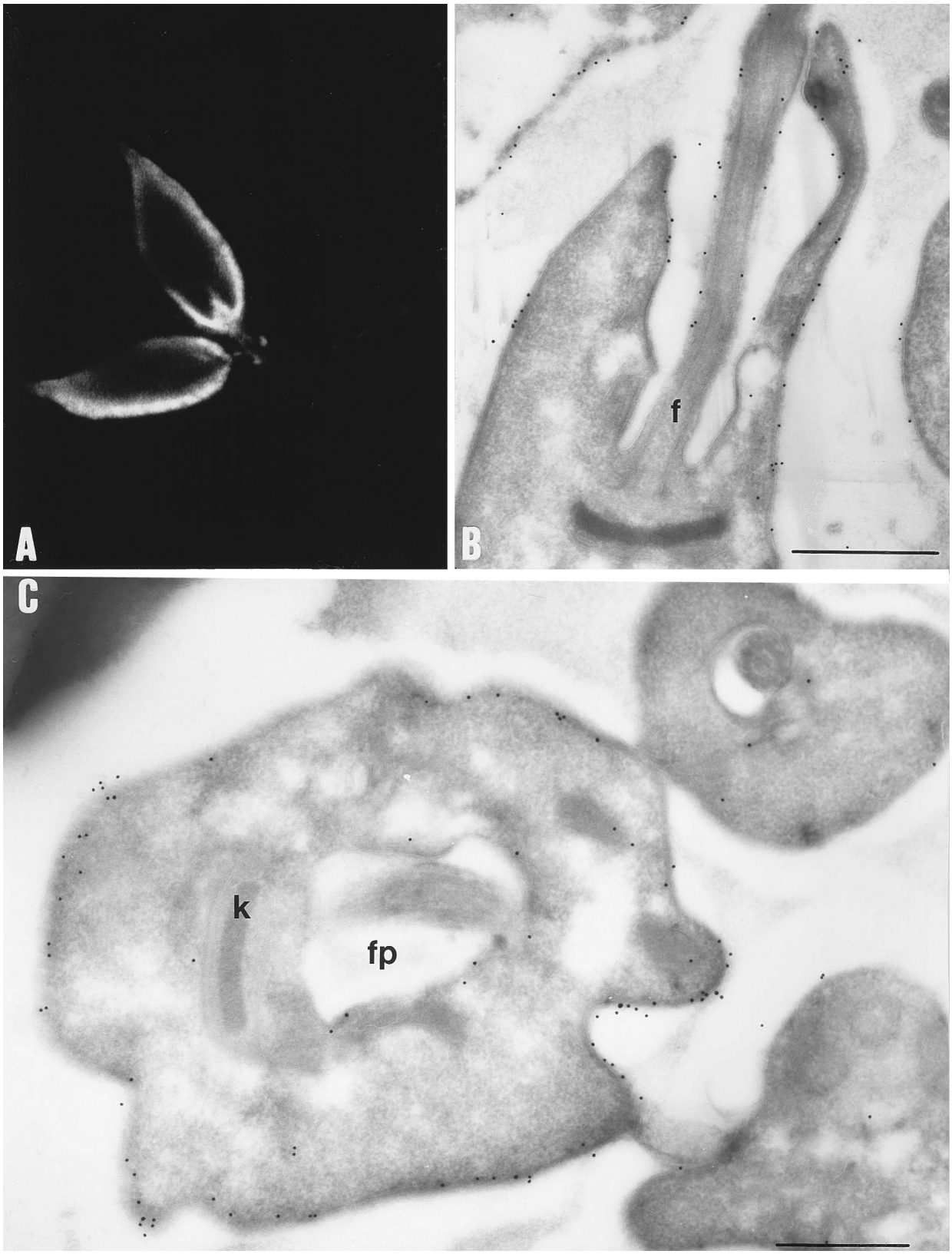

FIG. 8. Immunolocalizations of D1 transporter in D1pX.1 cells by using the affinity-purified D1-COOH antibody and confocal immunofluorescence microscopy (A) or immunoelectron microscopy of frozen thin sections (B and C). (B) Bar, 0.75 μm; f, flagellum. (C) Bar, 0.5 μm; fp, flagellar pocket; k, kinetoplast.

In addition, phosphatidylinositol 4,5-bisphosphate plays a central role in many signal transduction pathways by cleavage to inositol 1,4,5-trisphosphate and diacylglycerol (4). Phosphatidylinositol lipids are synthesized from inositol and diacyglycerol through CDP-diacylglycerol intermediates (35); hence, free inositol is an essential precursor for inositol phospholipid biosynthesis. Many organisms can synthesize inositol from glucose 6-phosphate, but cells often also possess specific membrane carriers that can transport inositol from the extracellular environment. Thus, yeast cells possess the enzymes for inositol biosynthesis and at least two inositol transporters, ITR1 and ITR2, whose genes have been cloned and sequenced (25). The deduced amino acid sequence of these two proteins reveals that they are related to mammalian facilitative glucose transporters and to structurally related active transporters, such as the *E. coli* arabinose/H^+ symporter. The transport of inositol in yeast cells is energy dependent (26), but the mechanism for coupling energy to transport has not been defined. Both the levels of the enzymes (11) and the level of the mRNA for one transporter (25) are reduced when inositol is added to the yeast growth medium. Mammalian cells such as kidney (14) and intestinal epithelium (7) and Ehrlich ascites tumor cells (16) possess active transporters for inositol. A gene for an Na^+/inositol cotransporter has been cloned from MDCK cells (18), and the deduced amino acid sequence of this protein is related to those of mammalian Na^+-dependent glucose transporters. The mRNA for this mammalian transporter is dramatically induced by hypertonic stress, and the transporter is believed to play a role in reestablishing isotonicity by accumulating inositol within the cell. Finally, some bacteria, such as *Aerobacter aerogenes* (12), possess inducible inositol transport systems and can utilize inositol as a carbon source.

Inositol transport has not been previously studied in the kinetoplastid protozoa. However, the extraordinary abundance of inositol phospholipids in the membranes of these parasites underscores the probable importance of the inositol phospholipid biosynthetic pathway and suggests that they may salvage the free alcohol, the phospholipids, or both from the extracellular medium. It is currently unclear whether the parasites can synthesize *myo*-inositol de novo from glucose 6-phosphate. However, yeast cells can both synthesize and transport *myo*-inositol, but this compound is still a growth factor (25) for these organisms, despite their ability to generate the alcohol enzymatically. Hence, the role and mechanism of inositol salvage from the environment by *Leishmania* parasites are worthy of investigation. In this paper, we have demonstrated that *L. donovani* promastigotes can transport *myo*-inositol across the plasma membrane by a carrier-mediated mechanism. The specific transporter responsible for the uptake is encoded by the previously cloned and sequenced D1 gene and is related in sequence to the yeast ITR1 and ITR2 transporters as well as many other members of the facilitative glucose transporter superfamily. Expression of this gene in *Xenopus* oocytes has been used to demonstrate the function and substrate specificity of the D1 protein and to confirm that it has kinetic properties and a pharmacology similar to those of the carrier measured in intact promastigotes.

One potential complication of studies which utilize a metabolizable substrate is that uptake measurements may be influenced by both transport and metabolism of the ligand. Specifically, such kinetic constants as K_m values derived from uptake measurements may be strongly influenced by the subsequent enzymatic steps of metabolism (36) if the transport step is not rate limiting. However, the K_ms determined for *myo*-inositol uptake in D1-expressing oocytes, wild-type parasites, and D1-overexpressing parasites are similar to the K_m derived from quantitation of transmembrane conductance (Fig. 6B), a property which is a direct measurement of the transport step. Consequently, these uptake measurements accurately reflect the transport step, which is apparently rate limiting.

We have quantitated the levels of D1 mRNA in promastigotes grown in the presence and absence of 25 mM *myo*-inositol by Northern blot analysis (data not shown) and have not observed any induction or repression of the message in the presence of substrate. Consequently, unlike the situation with yeast cells, the *Leishmania myo*-inositol transporter is not regulated by the level of extracellular inositol.

Mechanism of active transport by the D1 inositol transporter. The inositol transporters from both yeast and mammalian cells have been shown to be active carriers. Yeast cells are able to significantly concentrate free inositol within the cell (26), and the MDCK cell transporter is dependent upon Na^+ to concentrate the substrate when it is functionally expressed within oocytes (18). Experiments performed with *L. donovani* promastigotes or oocytes expressing the D1 gene demonstrate that uptake is strongly inhibited by such reagents as FCCP and DNP which collapse proton gradients but that inhibition of Na^+/K^+ ATPase or removal of Na^+ from the medium does not inhibit uptake. These results are consistent with the notion of the D1 protein functioning as a H^+ symporter, but they could also be explained by inhibition of inositol metabolism. Thus, if inositol transport is facilitative but uptake is enhanced by the trapping of such metabolic products as phosphatidylinositol within the cell, such drugs as FCCP and DNP which uncouple oxidative phosphorylation from ATP synthesis and thus attenuate metabolic processes could also inhibit net inositol uptake. In contrast, the detection of an inositol-dependent transmembrane current in D1-expressing oocytes is a direct demonstration that this protein functions as an electrogenic symporter. The further demonstration that transport is not dependent upon Na^+ and is promoted by increased H^+ concentration is consistent with the interpretation that the active transport process is coupled to proton translocation.

Previous studies have shown that *L. donovani* promastigotes possess a plasma membrane H^+-ATPase (42) and that they maintain an almost constant proton electrochemical gradient (43) over a range of extracellular pHs of 5 to 8 which can be utilized to drive active transport. It has been suggested that both glucose and proline transport (41) are coupled to this electrochemical gradient by proton symport, but other authors (37, 38) have contended that glucose and proline are transported by facilitative carriers. In previous experiments with the Pro-1 (21) and D2 (20) glucose transporters, we have been unable to detect glucose-dependent currents in oocytes expressing either of these two transporters; however, these experiments suffer from the uncertainty that they produce negative results. The present observation of proton coupling to inositol transport confirms that this carrier engages the proton electrochemical gradient for active transport.

Subcellular location of the D1 inositol transporter. The development of a transporter-specific polyclonal antibody has allowed us to monitor the distribution of the D1 protein by immunolocalization. Confocal immunofluorescence images of parasites overexpressing the D1 protein reveal abundant localization at the plasma membrane and a much lower level of punctate staining over the flagellum, whereas immunoelectron microscopy fails to detect as large a quantitative difference between these two organelles. For reasons enumerated previously (28), we believe that the immunofluorescence images provide a more accurate quantitative measure of the distribution. If this surmise is correct, the overexpressing parasites target the transporter more efficiently to the plasma membrane

than to the flagellum. Previous studies of the two isoforms of the Pro-1 glucose transporter reveal that isoform 1 is targeted primarily to the flagellar membrane and isoform 2 is targeted primarily to the plasma membrane, confirming the functional differences between these two organelle membranes. Similarly, the D2 protein is targeted to the plasma membrane but not the flagellum (20). Hence, it would not be surprising if the D1 protein should also show at least some differential distribution for these two organelles.

Importance of the D1 inositol transporter for parasite viability. It is currently unclear whether the D1 transporter is required for parasite viability or whether the parasites can acquire the necessary levels of inositol by de novo biosynthesis or by salvage of inositol phospholipids. Should these microorganisms depend upon salvage of this alcohol from the medium for efficient growth, this transport pathway might provide a useful target for development of novel chemotherapies. These issues may be addressed both by attempted gene knockout experiments (10) to test the viability of D1 null mutants and by investigations of the possible biosynthesis of inositol or of its transport in the bound form of inositol phospholipids.

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