STUDIES ON THE SUBCELLULAR LOCATION OF A GLUCOSE TRANSPORTER-LIKE PROTEIN IN *LEISHMANIA ENRIETTII*

by

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DEDICATION

To my parents, Cheng Xu and Mei-Zhen Pan, who have supported and encouraged me throughout my education.

To my husband, Yun-Cai Cai, who has not only been a source of strength in my graduate years, but has also been a source of support, encouragement, love and happiness whenever I needed it most.

To my motherland, The People's Republic of China, who provided my previous knowledge and development.

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ABBREVIATIONS

ANP, atrial natriuretic peptide;

C, carboxyl-terminus;

DME-L, Dulbecco's Modified Eagle-*Leishmania* ;

EDTA, ethylenedinitrilo-tetraacetic acid, disodium salt; ECL, enhanced chemiluminescence;

GLUT 1, isoform 1 of the human glucose transporter; gp 63, glycoprotein 63;

GPI, glycosyl phosphotidyl inositol; GTS, glucose transporter superfamily;

GST, glutathione S-transferase;

IAA, Indoleacrylic acid; IPTG, isopropyl-b-D-thiogalactopyranoside; iso-1, isoform-1;

kb, kilobase; kD, kilodalton; KLH, keyhole limpet hemocyanin;

L, Leishmania ; L, loop; LBA, Luria broth with ampicillin; LDL, low-density-lipoprotein;

LSB, Laemmli sample buffer;

MEM, minimal Eagle's medium;

N, amino-terminus;

PARP, procyclic acid repetitive protein; PCR, polymerase chain reaction;

pATH, plasmids are amenable for making Trp hybrids; PBS, phosphate buffered saline;

pGEX, plasmid expression vector(s); PMSF, phenylmethylsulfonyl fluoride;

RT, room-temperature;

SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis;

TrpE, anthranilate synthase;

VSG, variant surface glycoprotein;

MTPBS, 150 mM NaCl, 16 mM Na_2HPO_4, 4 mM NaH_2PO_4 (pH 7.3);

NET, 50 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.1% NP40, 0.25% gelatin;

TBS, 140 mM NaCl, 10 mM Tris, pH 7.4;

TBS-T, 0.02 M Tris, pH 7.6; 0.15 M NaCl, 0.05% Tween 20;

ABSTRACT

Leishmania has two life cycle stages: promastigotes, which live in the insect vector; and amastigotes which live inside the vertebrate host macrophages. In the process of its life cycle, the parasite undergoes morphological and biochemical transformations in order to adapt to the two very different environments of the insect vector and the vertebrate host. It must have certain stage-specific or developmentally regulated genes which are responsible for the different phenotypic properties of promastigotes and amastigotes. In this thesis, I have studied one such developmentally regulated gene, designated Pro-1, which is expressed in the promastigote stage of the life cycle and encodes a putative sugar transporter. The Pro-1 gene has seven to nine copies which are arranged in a single tandem repeat. This repeat encodes two closely related isoforms of the Pro-1 protein. The first copy of the Pro-1 gene encodes a distinct isoform (isoform 1) which contains a unique hydrophilic amino-terminal domain, compared to the other isoform (isoform 2) encoded by all other copies of the gene. The purpose of my work was to localize these two isoforms of the Pro-1 transporter. The best way to answer this question was to develop antibodies which can recognize isoform 1 and isoform 2. We have tried several different ways to raise antibodies against the Pro-1 polypeptide: 1) Antibody directed against a carboxy-terminal peptide of Pro-1; 2) Antisera against fusion proteins containing different regions of Pro-1; 3) Antisera against the whole Pro-1 protein using the recombinant vaccinia virus that expresses the Pro-1 protein. In addition, we have attempted epitope tagging, a method in which the C-terminus of Pro-1 is modified by the addition of a small peptide epitope (Asp Tyr Lys Asp Asp Asp Asp Lys). A commercially available monoclonal antibody which recognizes this epitope is then used to localize the modified Pro-1 protein.

INTRODUCTION

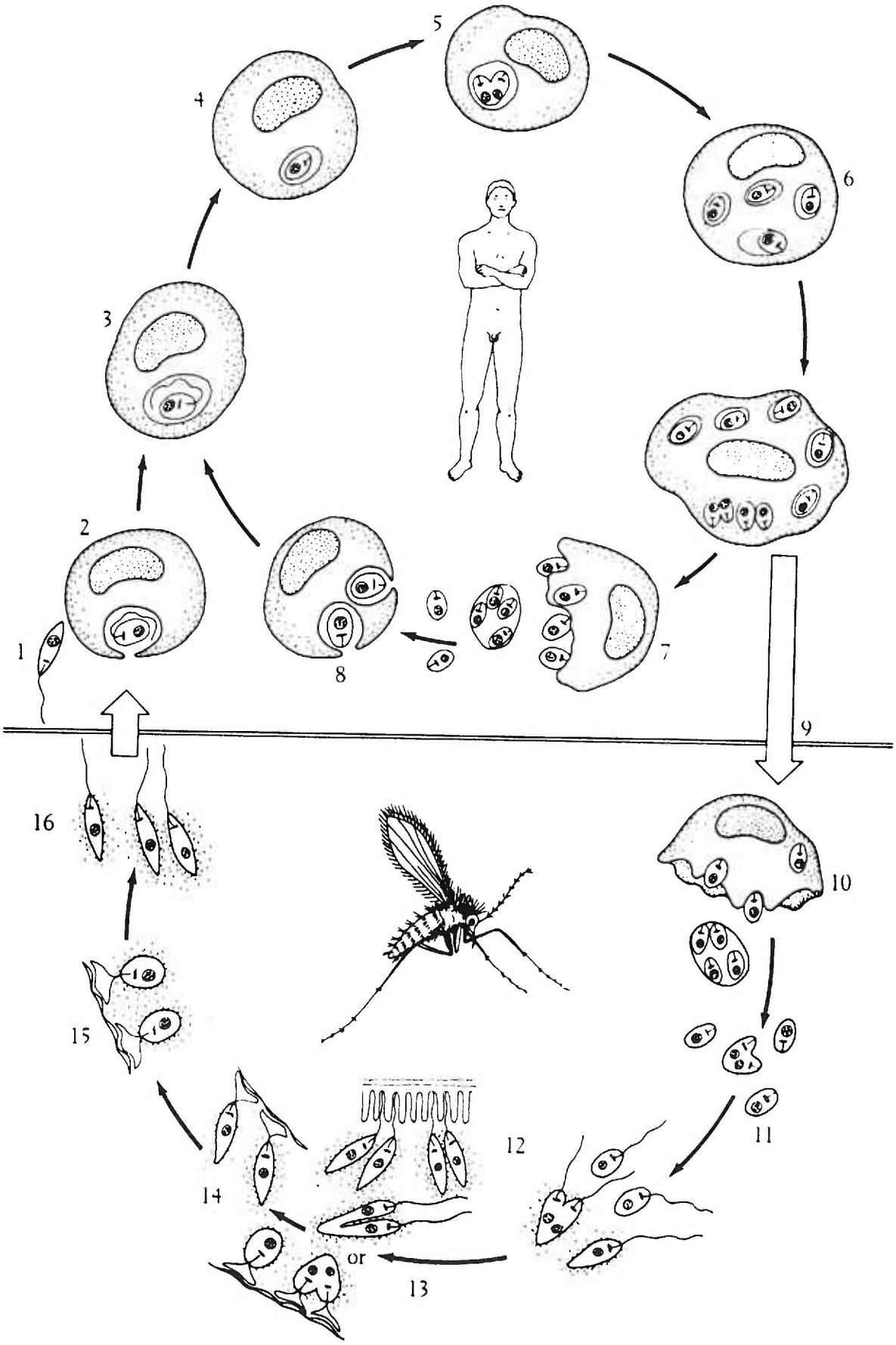
The Biology of *Leishmania*

1. Life Cycle

Leishmania are parasitic protozoa which are some of the most primitive eukaryotes (1). These parasites have two life cycle stages: promastigotes and amastigotes (Fig. 1). **Promastigotes** colonize the gut of the sandfly, the insect vector which transmits the infection from one vertebrate host to another, while **amastigotes** live within the macrophage lysosome of the mammalian host. When a sandfly bites an infected host and takes a blood meal, it ingests the parasite infected macrophages. Once inside the insect gut, the amastigotes are released from the macrophages and transform to the promastigotes. These promastigotes are elongated, flagellated, motile organisms. After colonizing the sandfly midgut, they migrate to the proboscis, where they transform from a non-infectious to an infectious form (called the metacyclic stage). When the infected sandfly bites another host and injects the infectious promastigotes into the host skin, they invade macrophages and transform back into amastigotes. The amastigotes are oval-shaped, non-motile, obligate intracellular organisms. These amastigotes replicate inside the macrophage, lyse the host cell, are released into the circulation, and reinvade other macrophages. The life cycle is completed when a sandfly bites an infected host again (2).

Promastigotes and amastigotes are adapted to very different physiological environments (3). Promastigotes colonize the sandfly gut whose pH is close to neutrality and whose temperature (about 26°C) is lower than that of the vertebrate host, while amastigotes live within lysosomes of the macrophages, where the pH is around 4.5 and the temperature is that of the warm blooded host (37°C) (3). In addition, the nutrients available to each life cycle form are different. The sandflies take sugar meals which are the primary source of

Figure 1. Life Cycle. *Leishmania* has two life cycle stages: promastigotes and amastigotes. Promastigotes live in the sandfly gut where they undergo a sequential differentiation from non-infective midgut forms [12-15] to infectious proboscis forms [16, also called the metacyclic forms] before the sandfly bites a host. The infection is initiated when these metacyclic forms are injected into the host, where they invade macrophages and convert into amastigotes [1-8]. These amastigotes replicate inside the macrophage, lyse the host cell, are released into the circulation, and reinvade other macrophages. When a sandfly bites an infected host, it takes up the infected macrophages. Once inside the insect gut, the amastigotes are released from the macrophages and transform to the promastigotes [10-12]. This figure has been reproduced from reference (2) with the permission of the publisher.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16
or

their nutrition (4) , so that the promastigotes are exposed to relatively higher sugar concentrations compared to the amastigotes. Promastigotes metabolize sugars such as glucose at a high rate (5). In contrast, intracellular amastigotes are not exposed to high sugar concentrations and metabolize glucose at a significantly lower rate than promastigotes. These amastigotes utilize fatty acids as their principal energy source (5, 6).

The need of the parasite to adapt to very different environments within the insect vector and the vertebrate host requires the expression of certain stage-specific or developmentally regulated genes which are responsible for the different phenotypic properties of promastigotes and amastigotes. In this thesis, I have studied one such developmentally regulated gene. This gene, designated Pro-1 (7), is expressed in the promastigote stage of the life cycle and encodes a putative sugar transporter. The expression of this putative sugar transporter is apparently restricted to the stage of the life cycle in which sugars are readily available for metabolic utilization.

2. The Disease

Leishmania parasites cause leishmaniasis, a disease that afflicts an estimated 17 million people around the world and is probably the second most important disease caused by parasitic protozoa (6). There are four major species of *Leishmania* that can infect human beings (8): *L. tropica*, which causes Old World cutaneous leishmaniasis (Oriental sore); L. *mexicana*, which causes New World cutaneous leishmaniasis; *L. braziliensis*, which causes New World mucocutaneous leishmaniasis; and *L. donovani*, which causes visceral leishmaniasis (kala-azar). However, *L. enriettii* , which is a non-pathogenic parasite to the human beings, has been used in many of our experiments, because it is quite safe for the laboratory workers.

Old World and New World cutaneous leishmaniasis is characterized by papules and nodules at the site of infection , producing ugly, large, dry, crusty lesions. Some lesions start with papules or nodules that tend to ulcerate and leave scars upon healing. If there isn't any secondary infection or trauma, lesions usually heal within 9 months.

New World mucocutaneous leishmaniasis consists of multiple lesions, such as chiclero ulcer, pian bois, uta espundia and etc. Some strains tend to invade the oronasal mucosa either initially by direct extension or by metastasis. It could be many years between the appearance of the primary and metastatic lesions. Secondary lesions occur on the skin as well as on mucous membranes. The oronasal mucosa lesions can lead to massive soft tissue and cartilage destruction of the face and larynx which can be fatal.

In visceral leishmaniasis, the parasites invade macrophages of the liver, spleen and bone marrow rather than the macrophages of the peripheral tissues. The first symptoms consist of malaise, headache, and fever which appear at irregular intervals. Untreated visceral leishmaniasis is usually a fatal disease, and death frequently occurs from complications due to secondary infections.

Project Background

One of the principal interests of our laboratory is to study genes that are developmentally regulated during the *Leishmania* life cycle. Identifying genes that are expressed preferentially or exclusively in one stage of the life cycle would give insight to the microorganism's adaptation to its changing milieu. Important questions concerning such developmentally regulated genes include i) the molecular mechanisms which govern their regulated expression, and ii) the biological functions of such genes in the parasite life cycle.

In order to identify genes that are specific to the promastigote stage, a cDNA library (9) was constructed in the vector λgt11 (10). A differential hybridizaton using rabiolabelled cDNA probes templated from either promastigote or amastigote polyadenylated RNA was performed. Autoradiograms of the filters were inspected for plaques that hybridized intensely to the radiolabelled promastigote cDNA probe but not to the amastigote probe. One of these clones, designated Pro-c1 (a cDNA clone) (7), provided the basis of the work in this thesis. A Northern blot of promastigote and amastigote RNA was probed with the Pro-c1; this blot (Fig. 2) revealed a 3.3-kb message which was much more abundant in the promastigote stage than the amastigote stage. Rehybridization of this Northern blot to a *Leishmania* rRNA clone (11) confirmed that equal amounts of RNA were loaded in each lane. This result demonstrates that this mRNA is developmentally regulated and is highly expressed in the promastigote or insect stage of *L. enriettii* (7). Consequently, the gene encoding this RNA was designated Pro-1. Subsequently, sequence analysis of the Pro-1 gene revealed that it encoded a protein similar to several known membrane transport proteins (12). These transport proteins fall into a superfamily (13) of transporters called the glucose transporter superfamily (GTS).

These integral membrane transporters include mammalian facilitative glucose transporters and a variety of other sugar transporters in both higher and lower eukaryotes and in bacteria (12). All of these proteins possess significant amino acid sequence similarity to one another. In addition, they possess similar predicted secondary structures containing twelve putative transmembrane a-helices, interconnected by hydrophilic loops on either side of the membrane (14). For the mammalian erythrocyte glucose transporter, the hydrophilic amino terminal and carboxy terminal tails have been localized (23, 24) to the cytoplasmic side of the membrane, and other members of this family probably have the same overall topology (Fig. 3). All of these proteins transport sugars, such as glucose, galactose, arabinose or

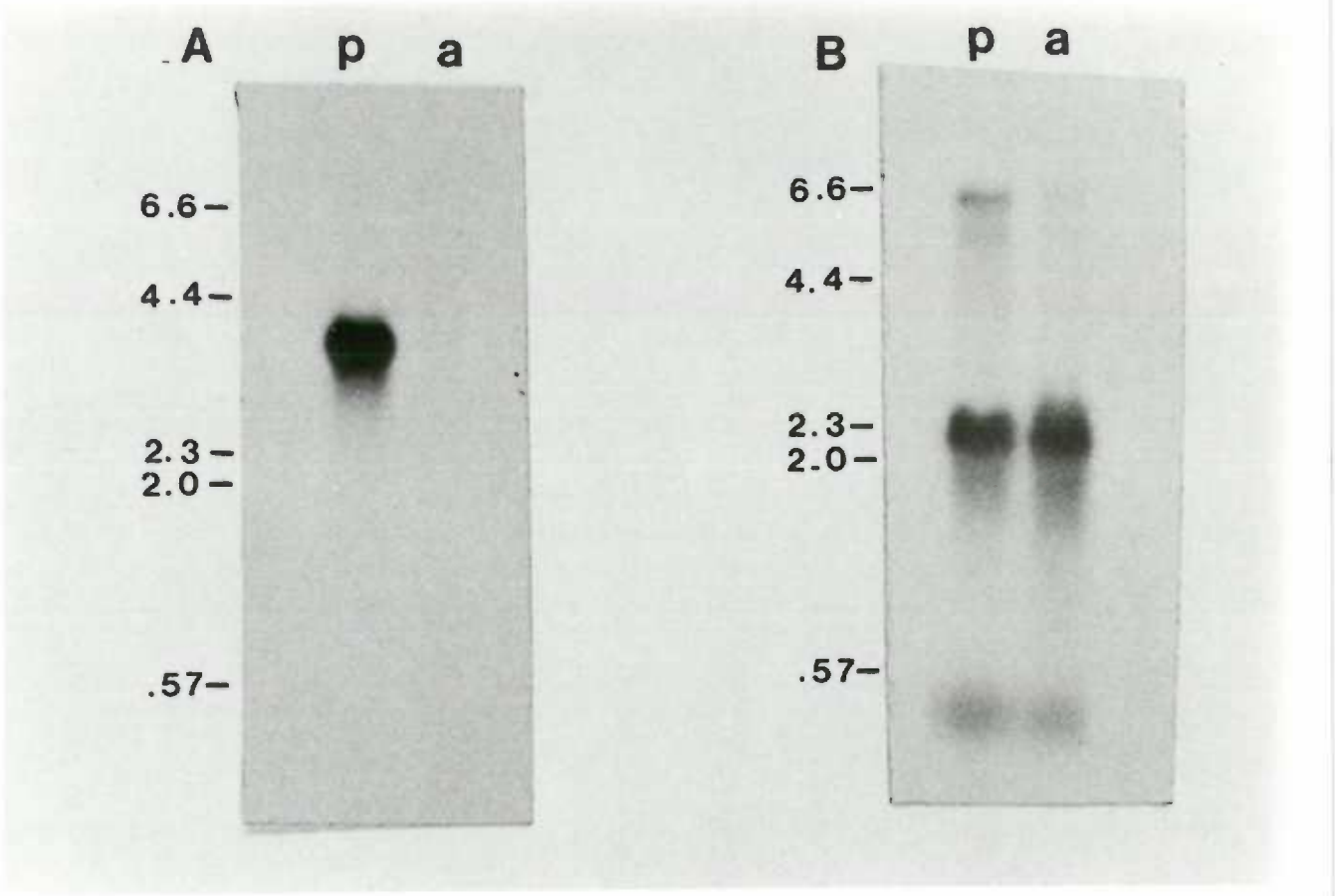
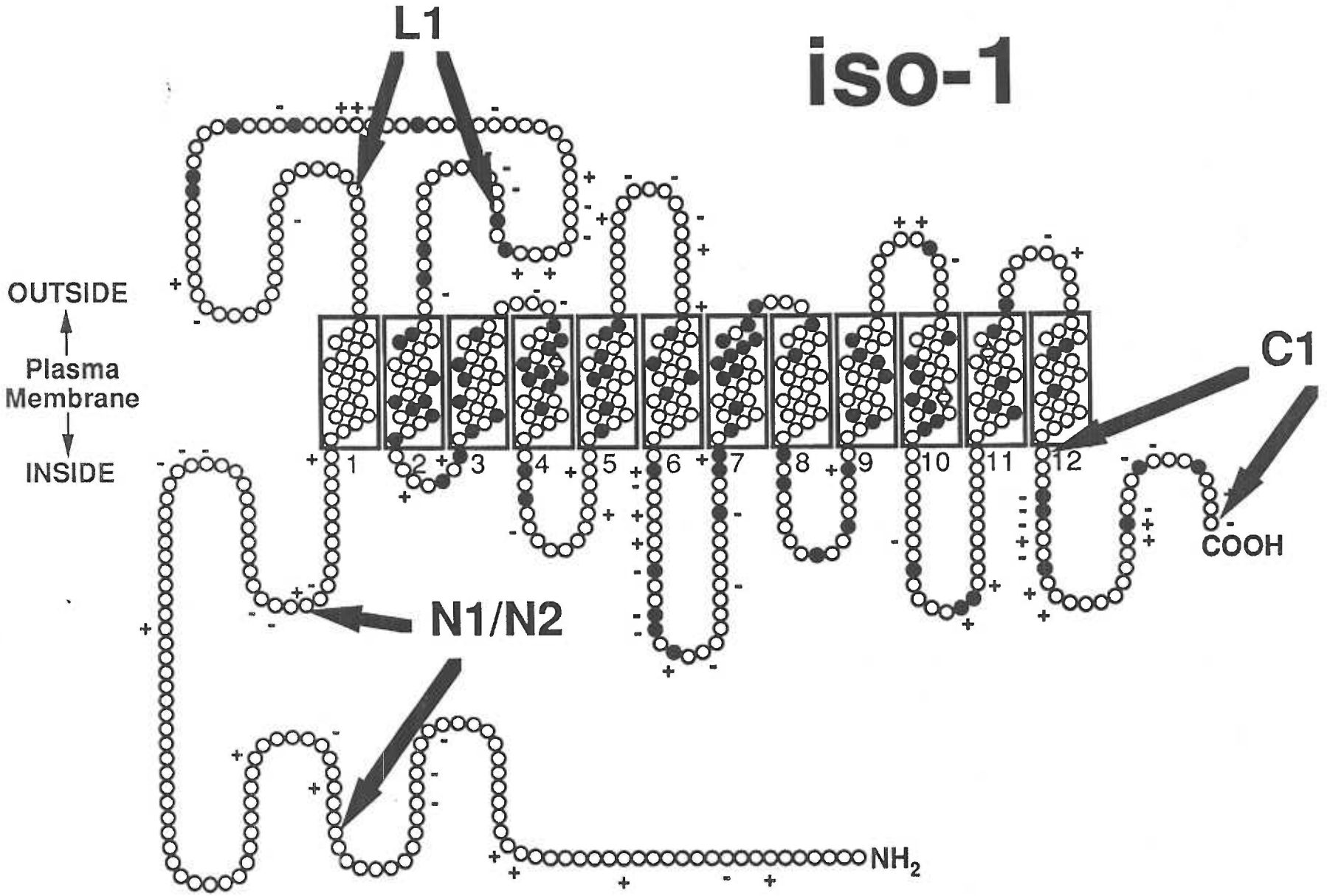


Figure 2. Northern blot of total RNA from promastigotes (lane p) and amastigotes (lane a). Total RNA was isolated from *L. enriettii* amastigotes grown in guinea pig lesions and promastigotes grown in tissue culture. Each lane contained 5 μg of total RNA and were electrophoresed on a 1% agarose-fomaldehyde gel, transferred to nitrocellulose and hybridized to the insert of the Pro-c1 cDNA clone (panel A) (6). The Pro-c1 probe was removed by boiling the blot in distilled water and the filter was rehybridized with a *L. enriettii* ribosomal RNA probe (panel B) (11) to demonstrate that equal amounts of total RNA had been loaded onto each lane.

Figure 3. The predicted topology of isoform 1 of the Pro-1 polypeptide showing putative cytoplasmic, transmembrane and extracellular domains, according to the model of Mueckler *et al.* (14) for the human erythrocyte glucose transporter. The filled dots are the identical amino acids to the human erythrocyte glucose transporter. N1, N2, L1 and C1 represent the different fusion proteins that were made from the unique amino terminus of iso-1 (N1 and N2), the first extracellular loop (L1) and the carboxyl terminus (C1), respectively. Arrows indicate the specific amino acids that were included in each fusion protein construct. Isoform 2 is identical to isoform 1, except for the amino terminal hydrophilic tail which is shorter (46 amino acids) and completely different in sequence (see Fig. 5). The locations of the predicted transmembrane domains were determined using the Eisenberg algorithm (15).


iso-1
L1
C1
N1/N2
OUTSIDE
Plasma Membrane
INSIDE
1
2
3
4
5
6
7
8
9
10
11
12
COOH
NH2

xylose. Some organisms contain multiple isoforms of one transporter. There are at least five isoforms of the human glucose transporter, designated GLUT 1 through GLUT 5, each of which has a different sequence and tissue distribution (13).

Several properties of the Pro-1 polypeptide support its classification as a member of the glucose transporter superfamily:

i) The Pro-1 polypeptide has significant sequence similarity to other GTS members including a 22% identity, and a 44% similarity if conservative substitutions are considered, to the human erythrocyte glucose transporter (7).

ii) The Pro-1 protein contains twelve predicted transmembrane segments (Fig.3), using the Eisenberg algorithm for assignment of putative transmembrane sequences (15).

iii) Several short sequences that are highly conserved among other GTS members are present in the Pro-1 polypeptide at the analogous position in the sequence (7, 16).

Although the sequence analysis clearly indicates that Pro-1 is a GTS family member, we do not yet know which ligand Pro-1 transports. Although the current evidence is consistent with the possibility that Pro-1 is a glucose transporter, it will be necessary to test the function of Pro-1 experimentally to prove that it is a transporter and to determine its ligand.

The Pro-1 gene is arranged in a single tandem repeat within the *Leishmania* genome (17), as are many of the other genes identified so far in trypanosomatidae. This cluster has seven to nine copies of the repeat unit. However, the first copy of the Pro-1 gene contains a unique hydrophilic amino-terminal domain (the entire hydrophilic amino terminal tail is different in this copy of the gene), whereas the remainder of the protein coding region of this first copy is identical to the internal unit of the repeat (Fig. 4 and unpublished data). This suggests that the first unit of the Pro-1 tandem repeat codes for a structural isoform of the Pro-1 transporter that is distinct from the protein products of the internal units of the repeat (18). Therefore,

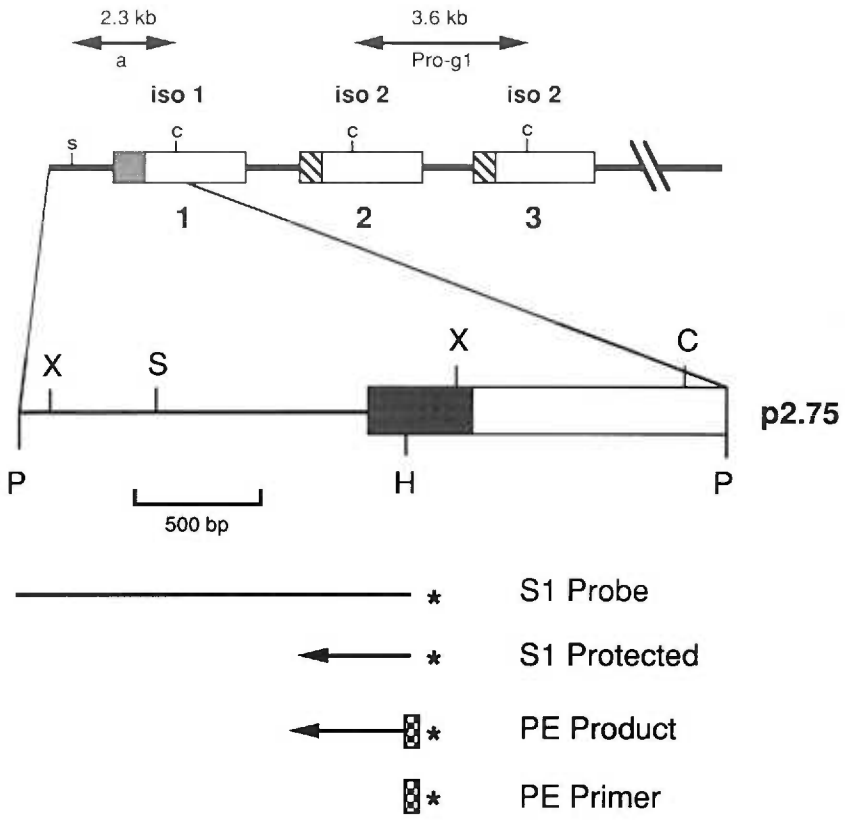


Figure 4. Chromosomal arrangement of Pro-1 transporter genes. The rectangles represent the protein-coding regions of the first three units of the tandem repeat. The stipled box represents the unique amino-terminal domain of the first copy (iso-1), and the cross-hatched boxes represent the amino-terminal domains of the internal copies of the gene (iso-2). There are approximately 7-9 units within the tandem repeat (17)

Pro-1 has two isoforms: iso-1 (the unique terminal copy in the repeat) and iso-2 (the internal copies in the repeat). One possible reason for the existence of two isoforms, which differ only in the amino terminal hydrophilic domain, is that each isoform might be targeted to a different subcellular location. For instance, iso-2 might be the major plasma membrane transporter, and iso-1 might be localized to an internal membrane bound organelle such as the glycosome (19). If this hypothesis is correct, it would indicate that one function of this amino terminal domain is to target the protein to its correct membrane address.

The glycosome is a unique organelle found in organisms of the order Kinetoplastida (19). It is a peculiar microbody-like organelle, resembling the peroxisomes of other eukaryotic cells, and it contains the glycolytic enzymes. To metabolize glucose, *Leishmania* parasites must transport this sugar across the plasma membrane and then across the glycosome membrane. Hence these parasites may require one glucose transporter in the plasma membrane (possibly iso-2) and another glucose transporter in the glycosomal membrane (possibly iso-1).

Alternative possibilities are i) that each isoform is located in a different subdomain of the plasma membrane, or ii) that both isoforms have the same subcellular distribution and that the function of the unique amino terminus does not involve differential targeting.

Specific Aims and Strategies

One primary objective of my research is to localize the two isoforms of the Pro-1 transporter. In the related parasite *Trypanosoma brucei*, two proteins involved in nutrient uptake, the transferrin receptor and the LDL receptor, are localized to a domain of the plasma membrane called the flagellar pocket (20). A third protein, of unknown function but with structural features similar to known receptors, is also localized to the flagellar

pocket (21). This invagination of the plasma membrane, which is attached to the base of the single flagellum, is thought to be a specialized organelle involved in the import of nutrients. One objective of the current work is to determine whether the Pro-1 transporter is also localized to the *Leishmania* flagellar pocket, or whether it is uniformly distributed over the plasma membrane. A second objective is to determine whether isoforms 1 and 2 are located in different places within the cell, e.g. whether iso-2 is a plasma membrane protein and iso-1 a glycosomal protein.

The best way to answer these questions is to develop antibodies which can recognize isoform 1 and isoform 2. We have tried three different ways to raise polyclonal antibodies against the Pro-1 polypeptide. In addition, we have attempted epitope tagging (22), a method in which the native protein is modified by the addition of a small peptide epitope. A commercially available monoclonal antibody which recognizes this epitope is then used to localize the protein.

1. Antibody directed against a carboxy-terminal peptide of Pro-1

The last 15 amino acids of the C-terminus of Pro-1 were synthesized, coupled to keyhole limpet hemocyanin (KLH) and injected into rabbits. The reason we chose the C-terminal peptide is because others have already successfully raised antibodies against C-terminal peptides from the human erythrocyte glucose transporter (23, 24). They found the antibody which is against the C-terminus can recognize the native protein, while an antibody which is against the N-terminus cannot recognize native protein, but will react on Western blots. The anti-peptide antibody approach was attempted by other members of the laboratory, before the initiation of this thesis project.

2. Antisera against fusion proteins containing different regions of Pro-1

Three different fusion proteins which contain either a fragment from the unique amino-terminus of iso-1 (N1 and N2, Fig. 3) or from the first extracellular loop (L1, Fig. 3) which is shared by both iso-1 and iso-2, were generated by cloning the specific fragment into an expression vector. These expression vectors produce a fusion protein containing the Pro-1 peptide fused onto the carboxy terminus of either the bacterial anthranilate synthase protein or the glutathione S-transferase protein from *Schistosoma mansoni*. In our experiments, the pATH and pGEX vectors were used to produce the appropriate fusion protein (25, 26).

3. Antisera against the whole Pro-1 protein

An alternative method, which has been used successfully to raise antisera against membrane proteins like the atrial natriuretic peptide (ANP) receptor (27), is to generate a recombinant vaccinia virus that expresses the protein of interest. A recombinant vaccinia virus that contains the entire Pro-1 coding region was prepared and inoculated into rabbits. The antisera obtained by this procedure should not distinguish between iso-1 and iso-2, but might be useful for determining the combined distribution of both isoforms.

4. Epitope Tagging Experiment.

A small hydrophilic peptide of eight amino acids (28), which is called the 'Flag' epitope (Asp Tyr Lys Asp Asp Asp Asp Lys), was engineered (see Materials and Methods) onto the C-terminus of Pro-1 (iso-2) by PCR mutagenesis. The Pro-1 gene containing the 'Flag' sequence was cloned into a *Leishmania enriettii* expression vector which is called pALT-Neo (29) and the transformants were selected on the drug G 418. A commercially available monoclonal antibody designated M2 (generous gift of Immunex Corp., Seattle, WA) that recognizes the 'Flag' epitope was used throughout this experiment.

All of the antibodies were tested by either Western blotting or immunoprecipitation. The ultimate goal is to develop specific antibodies which can be used in immunofluorescence, immunoelectron microscopy, and cellular fractionation to determine the subcellular location of each isoform.

MATERIALS AND METHODS

Growth of parasites and isolation of nucleic acids

Promastigotes of *Leishmania enriettii* were cultured at 26°C in DME-L (30) containing 10% heat-inactivated fetal bovine serum, 100 μM xanthine and 500 μg/ml hemin, and amastigotes were grown in male Hartley guinea pigs and isolated from the tissue lesions, as previously described (31, 32). Cells were collected at concentrations around 1 x 10^7 cells per ml, as determined using a hemacytometer counter. Genomic DNA and total RNA were isolated by phenol-chloroform extraction as detailed elsewhere (32, 33). Southern and Northern blotting were performed as described (33, 34). Inserts of plasmid clones were radiolabelled by the random oligonucleotide priming method (35). Hybridization of blots were performed at 42°C for at least 16 hr in 50% formamide, 5X SSC (1X SSC is 150 mM sodium chloride, 15 mM sodium citrate, pH 7.0), 10X Denhardt's solution, 20 mM sodium phosphate pH 6.5, 0.1% SDS, 250 μg/ml denatured herring sperm DNA. Filters were washed twice at room temperature and then three times at 55°C in 0.1 x SSC, 0.1% SDS.

DNA subcloning and expression

1. Generation of fusion proteins

i) TrpE fusion protein: The pATH expression vectors are plasmids designed to express chimeric anthranilate synthase (TrpE) fusion proteins from the E.*coli* tryptophan promoter-operator system (25). Each plasmid in the pATH series contains a polylinker cloning site which allows generation of a fusion onto the carboxy terminus of the TrpE protein in a particular reading frame. For construction of a fusion protein containing the unique amino

terminus of iso-1 (Fig.3), a 200 bp *Hind* III/*Xho* I fragment (1638 bp to 1838 bp of Fig. 5) was subcloned into the *Hind* III site of the pATH2 vector, using one internal *Hind* III site (at 1638 bp) and one polylinker *Hind* III site located downstream from the *Xho* I site. This 200 bp fragment encodes 67 amino acids, from Ser 52 to Arg 119 (Fig. 5) and a 44 kD fusion protein (designated **fusion protein N1**, where N indicates that the sequence is derived from the NH_2-terminus of iso-1) was induced when the plasmid was expressed in *E.coli* HB101. First , cells were grown in M9 medium (36) with tryptophan at 37°C for overnight. The following day, the cells were diluted 10-fold in M9 medium without tryptophan and shaken vigorously for 1 hr at 30°C. Then indolacrylic acid (IAA), which serves as an inducer of the Trp operon, was added and the fusion protein was induced between 4 hr to overnight. For screening clones, the cells were spun down and the cell pellet was resuspended in 50 µl cracking buffer (0.01 M sodium phosphate, pH 7.2; 1% β-mercaptoethonal; 1% SDS; 6 M urea). For a 100 ml culture of large scale TrpE fusion protein preparation, the cells were washed once in TE (10 mM Tris, pH 8.0; 1.0 mM EDTA) with 0.15 M NaCl and resuspended in 5.0 ml TE with 0.3 M NaCl. Then 5.0 mg lysozyme was added and the cells were left on ice for 30 min. Subsequently, 20% NP 40 was added to 1/100 of the total volume and the cells were incubated on ice for 15 min. Then NaCl-Mg solution (1.5 M NaCl, 12 mM $MgCl_2$) and 20 µg DNase I were added, and the cells were left on ice for another 15 min. The cells were spun down at 4°C for 15 min and washed in TE with 0.3 M NaCl and resuspended in 1.5 ml cracking buffer. Bromphenol blue was added to one tenth of the sample volume and the sample was analyzed on a 10% SDS-PAGE (37). The fusion protein band was cut out from the SDS-PAGE and minced. Subsequently the crushed gel was emulsified with Freund's Complete Adjuvant and injected into the rabbits.

ii) Glutathione S-transferase fusion proteins: The pGEX expression vectors are plasmids which have a tac promoter followed by the complete coding sequences of glutathione-S-

Figure 5. Sequence of the unique amino-terminus of iso-1 in the tandem repeat. Numbers at right indicate nucleotide sequences (top row) and amino acid position (bottom row). The deduced amino acid sequence of iso-1 is shown under its DNA sequence, while part of the deduced N-terminal amino acid sequence of iso-2 is shown in italics under the iso-1 amino acid sequences. The boxed amino acids are the predicted transmembrane a helices, as determined by the algorithm of Eisenberg *et al*. (15); the number beneath the box represents each transmembrane domain. N1, N2 and L1 represent three different fusion proteins that were made from the unique amino terminus of iso-1 and the extracellular loop which is common to the iso-1 and iso-2. Arrowheads indicate the beginning and the ending of the amino acids included in these fusion proteins. *Hind III*, *Xho* I and *Rsa* I indicate the locations of restriction sites used in the construction of the vectors which express each fusion protein.


Hind III
N1, N2
Xho I
N1, N2
Rsa I
L1
Rsa I
L1
1
2
3
4
5
6
T

transferase (GST) encoded by one of the species of *Schistosoma* (26). A polylinker placed within the region encoding the GST carboxy terminus allows the expression of C-terminal fusion proteins. One of the advantages of using this pGEX vector is that the fusion protein can be purified from crude bacterial lysates under non-denaturing conditions by affinity chromatography on commercially available glutathione agarose beads and then by competitive-elution with excess reduced glutathione. A 200 bp *Cla* I/*Sma* I fragment of the pATH vector encoding fusion protein N1, containing part of the unique amino-terminus (Fig. 3), was filled in using Klenow fragment of DNA polymerase I and subcloned into the *Sma* I site of the pGEX-3X expression vector. The resulting clone was transformed into XL-1 blue competent cells. The fusion protein was induced as follows: cells were grown in LB medium (36) with ampicillin (LBA) at 37°C overnight. The following day, the cells were diluted 10-fold in the same medium and shaken vigorously for 1 hr at 37°C. Then isopropyl-b-D-thiogalactopyranoside (IPTG), which acts as an inducer by inactivating the lacIq repressor, was added and a 33 kD fusion protein (designated **fusion protein N2**) was induced between 3 to 4 hrs. For screening clones, the cells were spun down and the cell pellet was resuspended in 1/50 to 1/100 culture volume of MTPBS (150 mM NaCl, 16 mM Na_2HPO4, 4 mM NaH_2PO4, pH 7.3). Cells were lysed on ice by mild sonication (15 sec x 2 with a small probe) and after adding Triton X-100 (Sigma) to 1%, were subjected to centrifugation at 10,000 x g for 5 min at 4°C. The supernatant was mixed with an equal volume of the 2 x Laemmli sample buffer (LSB) (36) and analyzed on SDS-PAGE. For affinity purification of fusion proteins, the supernatant was mixed with an equal volume of the pre-swollen 50% glutathione-agarose beads (sulphur linkage, Sigma) in MTPBS at room-temperature (RT) for 10 min on a rotating platform. After absorption, beads were collected by brief centrifugation at 500 x g and washed 3 x with MTPBS. Fusion protein was eluted by competition with free glutathione using 2 x 2 min washes with 1 bead volume of 50 mM Tris (pH 8.0) containing 5 mM reduced glutathione (Sigma) (final pH 7.5, freshly prepared). The large scale fusion protein preparation and purification were

exactly the same as the small scale preparation except that the volume of every reagent was proportional to the increased volume of the culture. Similarly, another 206 bp *Rsa* I fragment (1960 bp to 2166 bp of Fig. 5), which had undergone several intermediate subcloning steps to generate *Bam* HI sites at both sides, was cloned into the *Bam*-HI site of the pGEX-2T vector. This fragment comes from the first extracellular loop (Fig. 3) which is common to both iso-1 and iso-2 and encodes 68 amino acids, from Thr 160 to Tyr 227(Fig. 5). Since the fusion protein produced from this construct was degraded in XL-1 Blue cells, the construct was transformed into a *lon* protease deficient cell line which is called BL21(DE3) (38) and a 33 kD fusion protein (designated **fusion protein L1**, where L indicates that the sequence originates from the loop) was generated. The purified pGEX fusion proteins were emulsified in Freund's Complete Adjuvant and injected into the rabbits.

2. Generation of recombinant vaccinia virus.

A 3.3 kb fragment containing the entire Pro-1 protein coding region from the clone pX87 was cloned into the *Bam* HI site of the vaccinia expression vector pZVNeo. This construct was transfected into the BSC_{40} African green monkey kidney cell line which had been infected with vaccinia virus (39). Progeny virus were plaque purified and tested by hybridization, using a labelled probe of the Pro-1 gene, to detect viruses whose genomes had undergone recombination with the vaccinia sequences in the hybrid pZVNeo plasmid and hence had incorporated the Pro-1 gene into their genomes. These recombinant viruses contained the Pro-1 gene under the control of the vaccinia 7.5 K late promoter. Virus stocks were propagated by infecting BSC_{40} cells and purifying the virus over sucrose gradients (this work was done by Laurel Thomas, Vollum Institute for Advanced Biomedical Research, OHSU).

Preparation of total RNA from recombinant and non-recombinant vaccinia virus

BSC_{40} cells (1 x10^7) were infected either with wild type or recombinant vaccinia virus (5 pfu/cell) which contained the Pro-1 gene. Cells were incubated at 37°C for 4 hr and harvested by scraping. Cells were washed once with ice cold, sterile phosphate buffered saline (PBS) and lysed in prechilled denaturing solution (4 M guanidine isothiocyanate; 25 mM sodium citrate, pH 7.0; 0.5% sarcosyl; 0.1 M β-mercaptoethanol) followed by mild sonication on ice. RNA was extracted by adding 1/10 volume of 2 M sodium acetate, pH 4.0 and phenol /chloroform (5:1) and by spinning at 10,000 x g for 20 min at 4°C. The top aqueous phase was carefully removed, and an equal volume of isopropanol was added. The sample was kept at -20°C for at least 30 min to precipitate the RNA and pelleted by centrifugation at 10,000 x g for 15 min at 4°C. The RNA pellet was resuspend in 5 ml denaturing solution until the RNA was dissolved completely, mixed with an equal volume of isopropanol and precipitated as described above. The pellet was washed with ice-cold 75% ethanol and centrifuged as above. The pellet was dried in a vacuum dessicator and resuspended in RNase-free deionized H_2O.

Antisera preparation

1. Fusion Proteins

All the polyclonal antisera generated from the three different fusion proteins were prepared as follows: New Zealand White rabbits were injected subcutaneously with approximately 0.5mg/rabbit fusion protein emulsified with Freund's Complete Adjuvant. Four weeks after the first immunization, the rabbits were boosted with the same amount of fusion protein but emulsified with Freund's Incomplete Adjuvant. The rabbits were bled six weeks after the first immunization and subsequently at 2-week intervals, whereupon the rabbits were reimmunized in a similar manner. Rabbit blood was left at room temperature

for 2 hr in order to form a clot followed by centrifugation at 2500 rpm for 10 min to collect the serum. The serum was labelled and stored at -70°C. The serum used in later experiments was pre-absorbed with acetone powder which is an acetone extract of a cell line that only expresses either the anthranilate synthase or the glutathione-S-transferase. The induced (either pATH or pGEX) cell culture was spun down and resuspended in 2 ml of ice-cold 0.1M NaCl for every gram of cells. Four volumes of acetone (chilled to -20°C) were added and the suspension was mixed vigorously, incubated on ice for 1 hr, and spun down at 10,000 x g for 10 min at 4 °C. The pellet was resuspended in the same amount of acetone as above, recentrifuged, and dried in a vacuum dessicator. The sera (usually 1:10 dilution) was pre-absorbed with 10% acetone powder in TBS-T (0.02 M Tris, pH 7.6; 0.15 M NaCl, 0.05% Tween 20) at 4°C with a rotating platform overnight.

2. Vaccinia Virus

Rabbits were injected subcutaneously with 10^8 pfu of recombinant virus in 0.25 ml PBS. Animals were bled 21 day following the first injection and were then boosted with another injection of 10^8 pfu. The second bleed was performed 21 days after the booster injection. Serum was prepared as described above for the fusion protein injections.

Total promastigote protein and membrane lysates preparation

Total cell lysates were prepared by pelleting about 1 x 10^9 promastigotes, washing cells once in PBS and resuspending them in 100 µl 1 x LSB by heating at 95°C for 3 min. Lysates containing Triton X-114 soluble integral membrane proteins were prepared as follows: 1 x 10^{10} promastigotes were spun down and washed 2X in ice-cold TBS (140 mM NaCl, 10 mM Tris, pH 7.4). The cell pellet was resuspended in 10 ml TBS with 1% Triton X-114 and 1 mM $ZnSO_4$ and incubated on ice for 10 min followed by centrifugation at 50,000 x g at 4°C for 15 min. The supernatant was layered onto 15 ml of 6% sucrose in

TBS containing 1 mM $ZnSO_4$ and incubated at 30°C for 3 min to induce formation of a cloudy detergent phase. The detergent phase (about 400 µl) was collected by spinning at 1000 x g at RT for 10 min, mixed with equal volume of LSB and heated at 95°C for 3 min. Both total and Triton X-114 lysates were separated on a 10% SDS-PAGE.

Immunoblotting

Promastigote and amastigote lysates were separated on a 10% SDS-PAGE and electrophoretically transferred onto nitrocellulose filters (36). The nitrocellulose filters were first blocked with 5% nonfat milk in TBS-T for an hour followed by washing 5 x in TBS-T. Antibody reactions were done in the same buffer as above. The nitrocellulose strips were reacted with the rabbit antisera using dilutions ranging from 1:1000 to 1:10,000 by rocking 1 hour at room temperature or at 4°C. The strips were subsequently washed 5X in TBS-T. Following the primary antibody reaction, filters were treated with horseradish peroxidase-labelled goat anti-rabbit IgG (Bio-Rad) and the location of the peroxidase was revealed using the detection reagent Kit (Amersham) for enhanced chemiluminescence (ECL). The nitrocellulose strips were incubated in equal volumes of the detection reagent 1 and detection reagent 2 for 1 min and exposed to the Kodak XAR-5 film as required, usually from 5 sec to 1 min.

^{35}S-Methionine labelling and immunoprecipitation

Logarithmic phase promastigotes (5 x 10^6/ml x 100 ml) were washed twice with methionine-free minimal Eagle medium (MEM) and cultured in 10 ml of the same medium at room temperature on a rocker for 30 minutes to allow the parasites to metabolize their internal pools of Met. This medium was then removed and the cells were incubated with the same medium plus 1 mCi [^{35}S]methionine (Trans ^{35}S-label, NEN) for 4 hr at room

temperature on a rocker. The cells were washed twice in ice cold PBS and lysed with 1.0 ml triple detergent lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 0.1% SDS, 1% Nonidet P-40, 0.5% sodium deoxycholate) containing 1.0 mM phenylmethylsulfonyl fluoride (PMSF) and 1 µg/ml aprotinin, which were freshly added. For immunoprecipitation the lysates were first incubated with the pre-immune serum on ice for about 1 hr, then a suspension of Protein A (Sigma, formalin treated suspension of *Staphylococcus aureus*) was added to one tenth of the total volume and incubated on ice for another 0.5 hr. The suspension was spun down and the supernatant was incubated with immune serum in a 1:500 dilution for 1 hr on ice, then 25 µl pre-swollen protein A-Sepharose (Sigma) was added, and samples were rocked for an additional hour at 4°C followed by centrifugation at 12,000 x g for 2 min. Samples were then aspirated and pellets were washed two times (20 min, 4°C) with NET buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.1% NP40, 0.25% gelatin) and one time with 10 mM Tris pH 7.5, 0.1% NP40. Protein A sepharose pellets were then resuspended in 30 µl of LSB and the supernatants analyzed by SDS-PAGE.

Construction of plasmid encoding epitope tagged Pro-1 protein

The cloned gene encoding the Pro-1 protein was modified by PCR mutagenesis to attach the "Flag" epitope (Asp Tyr Lys Asp Asp Asp Asp Lys) onto the carboxy terminus. For these experiments, we began with the clone Pro-g1 which contains one unit of the tandem repeat (7). We designed the forward primer 5'- TG ACG ACG CTT GCA TCG ATT -3' (1485 bp to 1504 bp of Pro-g1) which contains the *Cla* I site that occurs in the middle of the protein coding region of Pro-1. The reverse primer 5'- GCTA TCGCGA TCA CTT GTC GTC GTC GTC CTT GTA GTC CTC AGC CCT GTT CCC GAT GG -3' is complementary to the last 20 nucleotides of protein coding region of Pro-1 (2003 bp to

2023 bp of Pro-g1) and contains the "Flag" epitope sequences and an *Nru* I restriction site. PCR reactions consisted of 30 cycles performed on a Techne PHC-1 thermocycler at a melting temperature of 95°C (0.5 min), an annealing temperature of 45°C (1 min), and an extension temperature of 72°C (1 min) followed by 5 min at 72°C. The reaction product (600 bp) was separated on 1% agarose gel and subcloned into the pANB87, which is a clone containing the whole Pro-1 protein coding region in the *Leishmania* expression vector pALT-Neo (29). However, the sequences analysis of the resulting clone showed that only part of the "Flag" sequence had been attached onto the C-terminus of the Pro-1 protein in this construct. Presumably, a portion of the "Flag" sequence had been deleted in the process of subcloning the PCR product. Therefore, we redesigned the reverse primer 5'- GCTACTGA GGATCC TCA CTT GTC GTC GTC GTC CTT GTA GTC CTC AGC -3' which contains the *Bam* HI site instead of *Nru* I site (*Nru* I produce a blunt ended restriction fragment and may have prevented efficient cloning of the PCR product) and "Flag" epitope sequences as well. Again, this 600 bp PCR product was digested with *Cla* I and *Bam* HI and subcloned into pANB87. The resulting clone, designated pAN87-Flag, can be transfected into *Leishmania* parasites, where it should express the Pro-1 protein containing the "Flag" epitope on its C-terminus.

DNA sequence analysis

All the above constructs, including the fusion protein and the epitope tagged Pro-1, were further confirmed by DNA sequencing. Single or double stranded DNA were sequenced by the dideoxy-chain termination method (40), using ^{35}S-dATP (New England Nuclear, Boston, MA) and the Sequenase Kit (United States Biochemical Corp., Cleveland, OH). Single-stranded plasmid DNA from Bluescript clones was prepared with the KO7 helper phage (41) infection and double-stranded plasmid DNA was prepared by the CsCl method, as previously described (36).

Transfection of *Leishmania* cells

Midlogarithmic cultures (5 x 10^6/ml) of *L. donovani* were harvested by centrifugation (1000 x g for 5 min). Cells were washed twice in 10 ml of ice cold PBS and resuspended in high ionic concentration electroporation buffer (21 mM Hepes, pH 7.5, 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 6 mM glucose) to a final concentration of 10^8 cells per ml. Cells (0.5 ml) were mixed with plasmid DNA (50 µg) , transferred to an electroporation cuvette, and exposed to an exponential discharge of 2250 V/cm from a 500-µF capacitor (Bio-Rad Gene Pulser) (29, 42). Electroporated cells were incubated on ice for 10 min, transferred into culture medium, and incubated 24 hr before adding the drug G418. The concentration of G418 was started at 10 µg/ml and gradually increased to 200 µg/ml.

Immunofluorescence

Promastigotes (10^6) of L. *donovani* transfected with pALT-Neo and pANB87-Flag were spotted onto glass cover slips, air dried, and then fixed in methanol for 20 min at -20°C. The cells were washed 2 x in PBS with 2% BSA, 0.1% Triton X-100. The cells were then blocked with 2% normal goat serum in PBS with 1% Triton X-100 followed by washing 4 x for 5 min each with the same buffer as above. The primary antibody reactions were done in several different dilutions, from 1:1000 to 1:10,000 in 2% BSA-PBS for 0.5 hr at room temperature followed by the same washing procedure described above. The goat anti-mouse rhodamine-B conjugated antibody (TAGO, Inc.) was applied at 1:100 dilution in 2% BSA-PBS at room temperature for 0.5 hr. The cells were washed 2 x for 5 min each in PBS with 2% BSA and then 3 x in PBS. The cover slips containing the cells were mounted in one drop of PBS with 50% glycerol and sealed with nail polish. Subsequently, the slides were examined under the fluorescence microscope (Leitz vario orthomat 2 Dialux 22EB) at an excitation wavelength of 530-560 nm. Several different conditions for fixation

such as 3% paraformaldehyde or 3.7% formaldehyde for 2 hr at either -20°C or 4°C or R.T. or 37°C, were tried in this experiment in order to reduce the background fluorescence observed in all samples.

RESULTS

In the experiments reported here, I have used three different approaches to raise antibodies against the Pro-1 polypeptide: 1) generation of antibodies against fusion proteins; 2) generation of antibodies against a recombinant vaccinia virus; 3) use of epitope tagging to modify the Pro-1 protein so that it can be detected with a commercially available antibody.

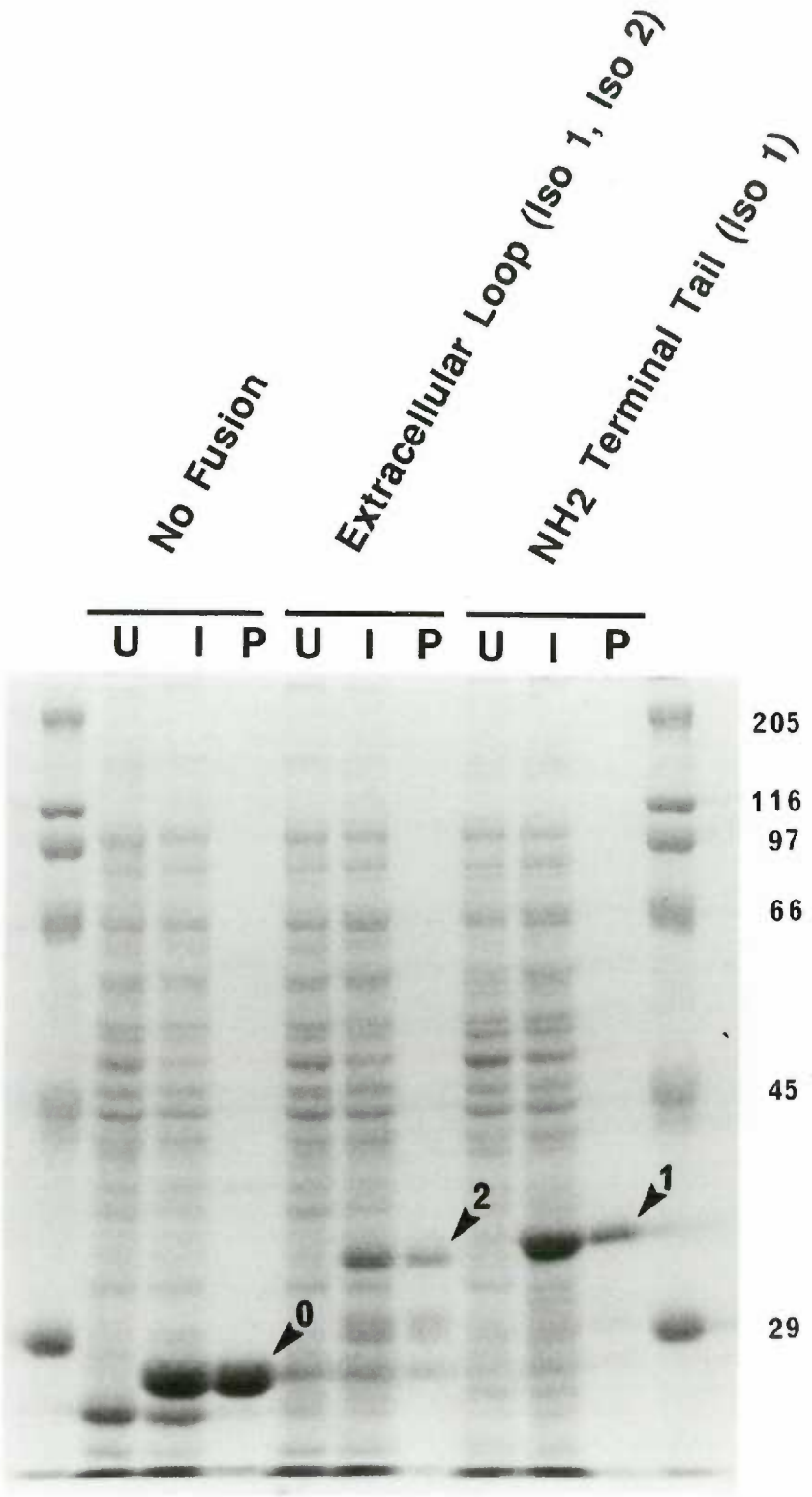
Fusion Proteins

In this approach, we have generated fusion proteins which contain different regions of Pro-1. The segments of Pro-1 contained in these fusion proteins were chosen to produce antibodies which will distinguish between iso-1 and iso-2 (Fig. 3).

We first tried to generate a fusion protein containing a fragment from the unique amino-terminus of iso-1. A 200 bp *Hind* III/*Xho* I fragment (1638 bp to 1838 bp of Fig. 5) was subcloned into the *Hind* III site of the pATH2 vector, using one internal *Hind* III site (at 1638 bp) and one polylinker *Hind* III site located downstream from the *Xho* I site. This 200 bp fragment encodes 67 amino acids, from Ser 52 to Arg 119 (Fig. 5) and a 44 kD fusion protein, designated N1, was induced when the plasmid was expressed in *E.coli* HB101. This fragment was also subcloned into the pGEX-3X expression vector to generate another fusion protein (N2) with glutathione S-transferase (Fig. 6, arrowhead 2) Both fusion proteins were then inoculated into rabbits to raise an antiserum specific for the unique amino terminus of iso-1. The segment of isoform 1 used to generate fusion proteins N1 and N2 is indicated in Figure 3 by the arrows marked N1/N2.

Figure 6. Expression and purification of GST and GST fusion proteins. Samples were taken from uninduced (U), induced (I), and glutathione agarose purified (P) lysates, which were size separated by 10% SDS-PAGE with Coomassie blue staining. The purified GST (26 kD), purified N2 fusion protein (33 kD) and L1 fusion protein (33 kD) were marked with arrowheads and the numbers 0, 1, and 2, respectively. Expression of GST and fusion protein: an overnight culture of cells transformed either with a pGEX-2T or pGEX-3X construct was diluted 1:10 in fresh medium, grown for 1hr and IPTG added to 4 mM. The uninduced culture was grown as above except there was no addition of IPTG. GST was purified with glutathione-agarose beads and eluted from beads (see materials and methods). The position and sizes (kD) of molecular weight markers are indicated at the right.

Glutathione S-transferase Fusion Proteins



Several approaches were attempted to generate an antibody that would recognize regions of Pro-1 common to both iso-1 and iso-2. Previously, others in our laboratory had attempted to raise antibodies against a C-terminal pentadecapeptide coupled to KLH using both dicyclohexylcarbodiimide and glutaraldehyde as coupling reagents. Neither of these two antisera recognized a polypeptide on Western blots. Subsequent attempts were made to prepare TrpE fusion proteins containing either the entire Pro-1 coding region or various portions of this sequence containing either single or multiple transmembrane segments. None of these constructs produced stable fusion proteins; the fusion proteins were either degraded to a protein of the same size as the native TrpE protein, by digestion of the Pro-1 segment of the fusion, or they were degraded to small polypeptides. Consequently, in this work we attempted to raise fusion proteins against exclusively hydrophilic segments of Pro-1. The inability to raise stable fusion proteins against the transmembrane regions suggested the possibility of raising a fusion protein against the first extracellular loop (Fig. 3, L1) which is common to both iso-1 and iso-2 and is also a hydrophilic fragment. Consequently, a 206 bp *Rsa* I fragment (1960 bp to 2166 bp of Fig. 5), which had undergone several intermediate subcloning steps to generate *Bam* HI sites at both sides, was cloned into the *Bam* HI site of the pGEX-2T vector. This fragment encodes 68 amino acids, from Thr 160 to Tyr 227 (Fig. 3 and Fig. 5, regions marked L1), and a 33 kD fusion protein, designated L1, was produced in a *lon* protease deficient cell line (Fig. 6, arrowhead 2). DNA sequence was obtained from the regions of these constructs encoding the fusion junction to ensure that the correct fragment had been cloned in frame with the TrpE or glutathione S-transferase polypeptide.

The fusion proteins were analyzed on the 10% SDS-PAGE (Fig. 6). The induced cells (I) contained fusion proteins of 33 kD absent in uninduced cells (U). These proteins are larger than the induced GST protein (26 kD) by the predicted amount for a protein containing a fusion of 67 amino acids for N2 and 68 amino acids for L1.

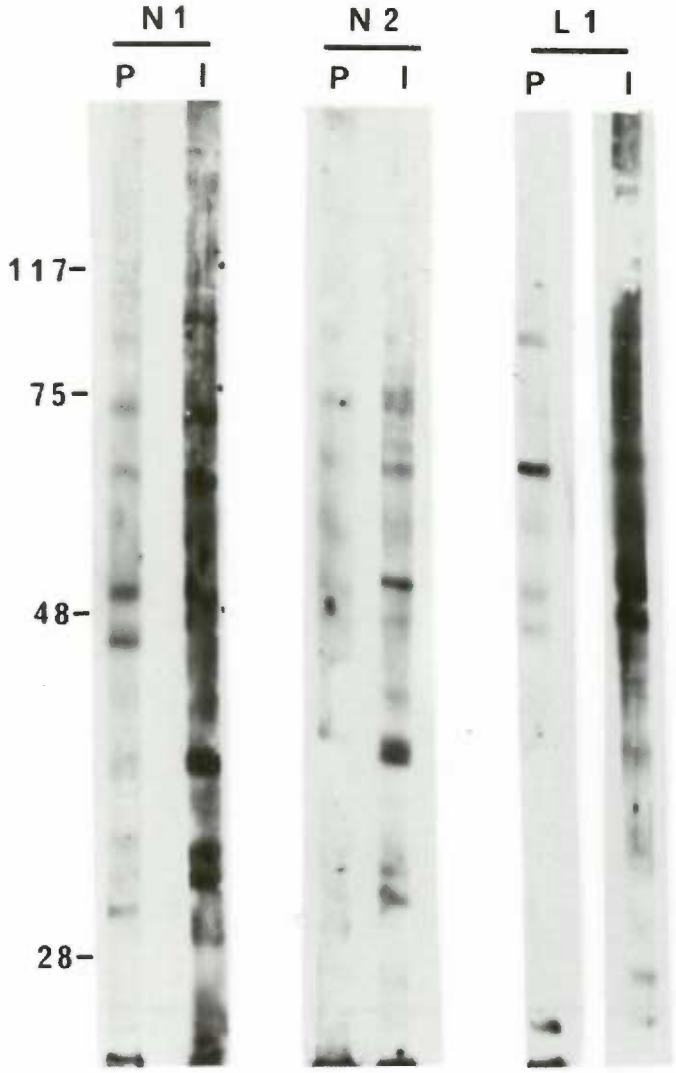
All three fusion proteins were inoculated into rabbits to generate three different polyclonal antisera. These antisera were then pre-absorbed with an acetone powder made from cells which expressed either the native TrpE or the native glutathione S-transferase protein. This pre-absorption step should help to remove antibodies that are directed against bacterial proteins. Each antiserum was then tested on Western blots to determine whether it recognized a specific polypeptide in the *Leishmania* lysates. If an antiserum does react with the Pro-1 polypeptide, there should be a band detected by immune serum, but not by pre-immune serum. This band would probably be enriched in a Triton X-114 lysate, since this fractionation procedure enriches for membrane proteins (43, 44). *Leishmania* total protein lysates and Triton X-114 membrane lysates of promastigotes were run on 10% SDS-PAGE and electrophoretically transferred onto nitrocellulose filters. Since Pro-1 is a putative membrane transporter, we expected that some specific bands would show up in the Triton X-114 lysates of the Western blot if these sera do contain antibodies directed against the Pro-1 protein.

Western blots of total lysates and Triton X-114 lysates probed with the N1, N2 and L1 antisera are shown in Figure 7. Although there are some discrete bands above a diffuse background on the strips probed with immune serum, most of these bands are also present in the pre-immune blots. Furthermore, there are no bands on these blots of total lysates which are enhanced in blots of Triton X-114 lysates, as would be expected for an antiserum which reacts with an integral membrane protein. In summary, these Western blots do not provide any convincing evidence that any one of these antisera recognize the Pro-1 polypeptide.

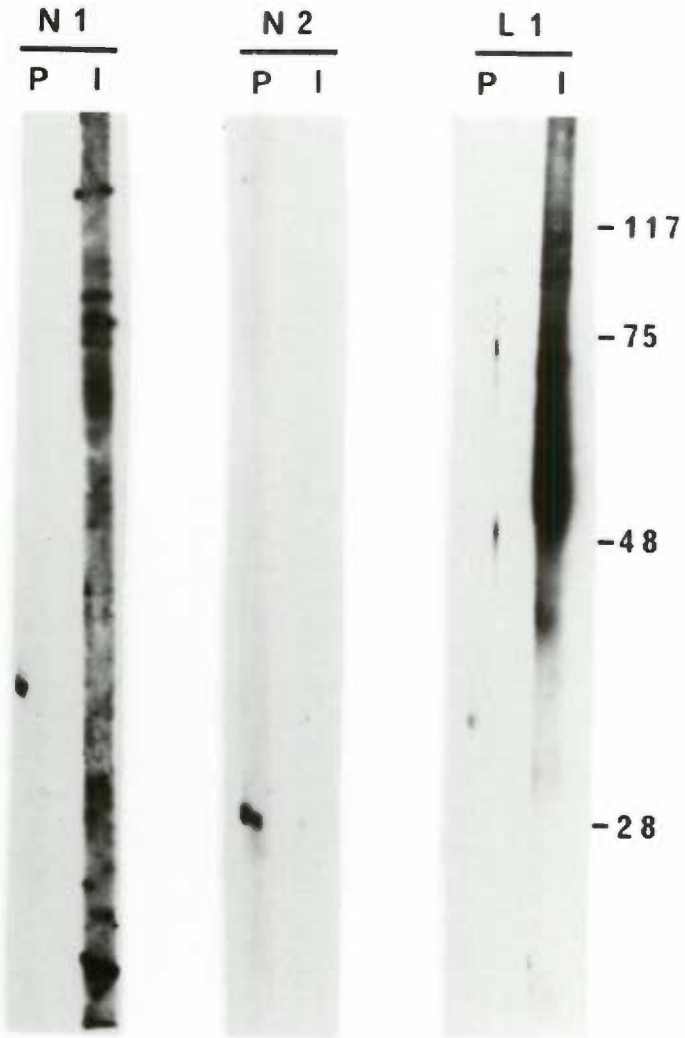
By contrast, antiserum raised against a C-terminal fusion protein derived from a different *Leishmania* transporter, designated the D1 transporter (45), does generate a discrete band

Figure 7. Western blots of promastigote lysates using antisera raised against the N1, N2 and L1 fusion proteins. Total protein lysates and Triton X-114 membrane lysates of promastigotes were separated in 10% SDS-PAGE and transferred to nitrocellulose filters. The nitrocellulose strips were reacted with pre-immune sera (P), and with three different immune sera (I) from three different fusion proteins, designated N1, N2 and L1. Numbers at far left and far right are molecular weight markers (kD).

Total Lysates



Triton X-114 Lysates



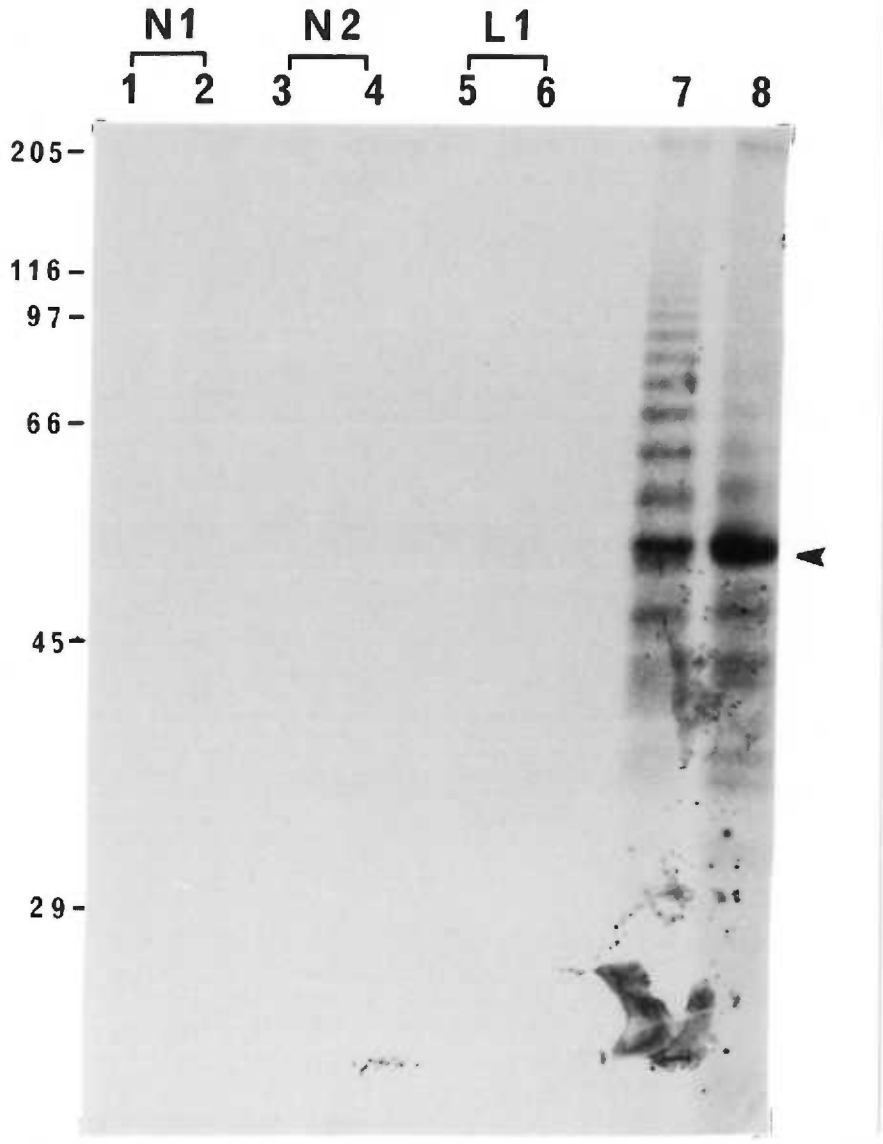


Figure 8. Immunoprecipitation of lysates from *in vivo* labelled promastigotes, using antisera raised against the N1, N2 and L1 fusion proteins. Cultures of logarithmic phase promastigotes of wild type *L. enriettii* (lane 1-6) and of *L. donovani* transfected with either pALT-Neo (lane 7) or pAN87-Flag (lane 8) were labeled with [^{35}S] methionine for 4 hr at room temperature. Immunoprecipitations were performed with pre-immune sera (lane 1, 3, 5; 1:500), with three different immune sera (lane 2, 4, 6; 1:500) from fusion proteins N1, N2 and L1, and with the M2 monoclonal antibody (lane 7, 8). The immunoprecipitates were analyzed by 10% SDS-PAGE followed by the PPO enhancing procedure (36).

were infected with this recombinant virus, and RNA was isolated from the infected cells. A Northern blot probed with the insert of the Pro-1 protein coding region demonstrates that the recombinant vaccinia virus does expresses RNA encoding Pro-1 (Fig. 9, lane 2), whereas cells infected with wild type vaccinia do not express this RNA (Fig. 9, lane 3). Methylene blue (0.02% methylene blue, 0.3M NaAc, pH 5.2) staining of this Northern blot shows that equal amounts of RNA were loaded in each lane. Subsequently, this recombinant virus was used to infect a rabbit and sera were collected two weeks after the initial infection and two weeks after each of two booster infections. These three bleedings were first tested by Western blot (Fig. 10), using both total protein from promastigotes and Triton X-114 membrane lysates. We also included total lysates from *L. enriettii* amastigotes, which should not express the Pro-1 protein, since there is very little Pro-1 mRNA expressed in amastigotes (7). If antibodies which recognize Pro-1 are present in the antisera, we would expect to observe a band which is present in both total and Triton X-114 lysates but which is absent from the amastigote lysates and from lysates probed with pre-immune serum. However, we do not detect a band with these properties on the Western blots.

In addition, the antisera were tested by immunoprecipitation of extracts from ^{35}S-methionine labelled *L. enriettii* promastigotes. The gel (Fig. 11) showed that all bands present in the immunoprecipitations of the three immune sera were also present in precipitations using pre-immune serum. A positive control was performed using an antiserum generated from recombinant vaccinia virus containing the extracellular and membrane anchor part of the atrial natriuretic peptide (ANP) receptor (courtesy of Dr. Michael Chinkers). ^{35}S-methionine labelled lysates of a human epithelial carcinoma cell line expressing the ANP receptor were immunoprecipitated with this antiserum. These control immunoprecipitations produced two bands of approximately 132 kD, the expected size of the ANP receptor, which were not present in the pre-immune precipitation. Once again,

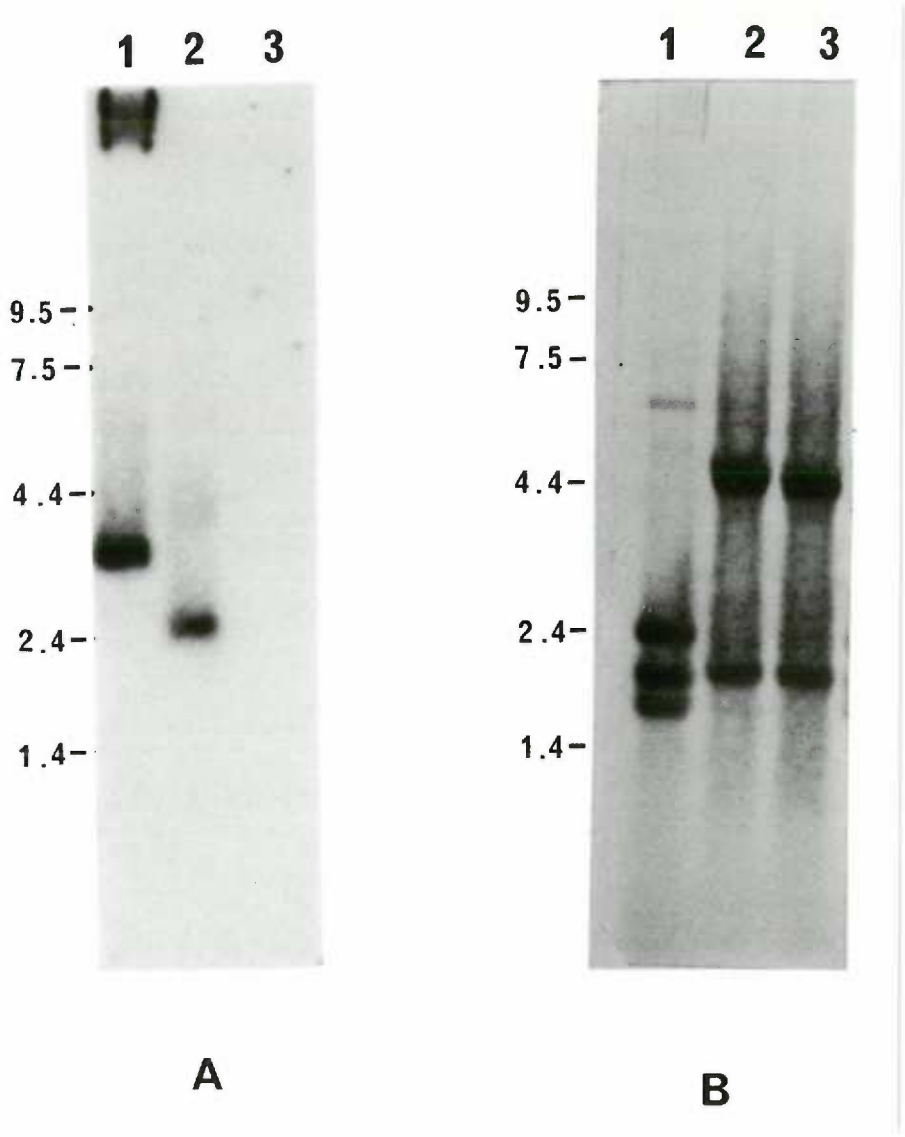


Figure 9. Expression of the Pro-1 gene in recombinant vaccinia virus. Total RNA was isolated from BSC_{40} cells which were infected with either wild type or recombinant vaccinia virus (5 pfu/cell) containing the Pro-1 gene. Five μg total RNA from promastigotes (lane 1), from cells infected with recombinant vaccinia virus containing the entire Pro-1 coding region (lane 2), from cells infected with wild type vaccinia virus (lane 3), were electrophoresed on a 1% agarose-formaldehyde gel and transferred to nitrocellulose. The Northern blot was first stained with methylene blue (panel B) to visualize total RNA. The methylene blue was then removed, and the filter was probed with the insert of the Pro-1 protein coding region (panel A).

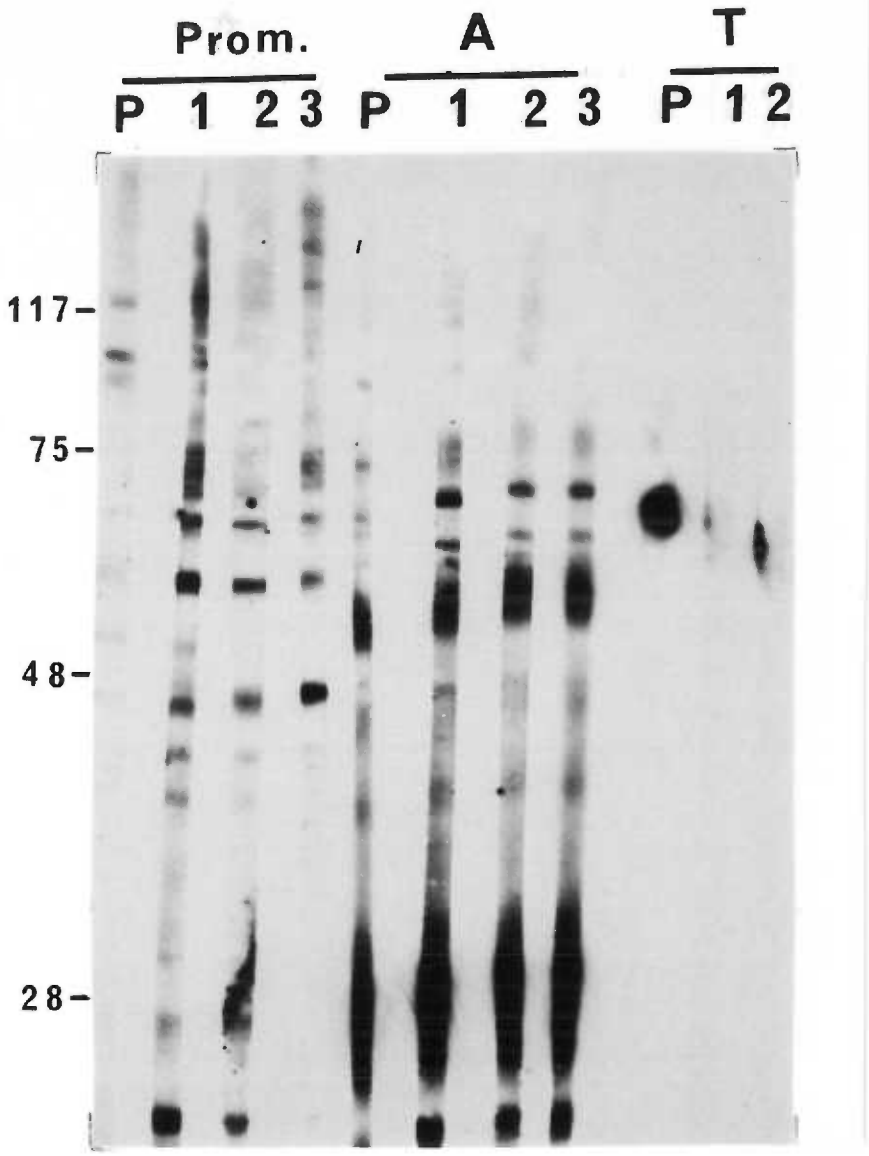
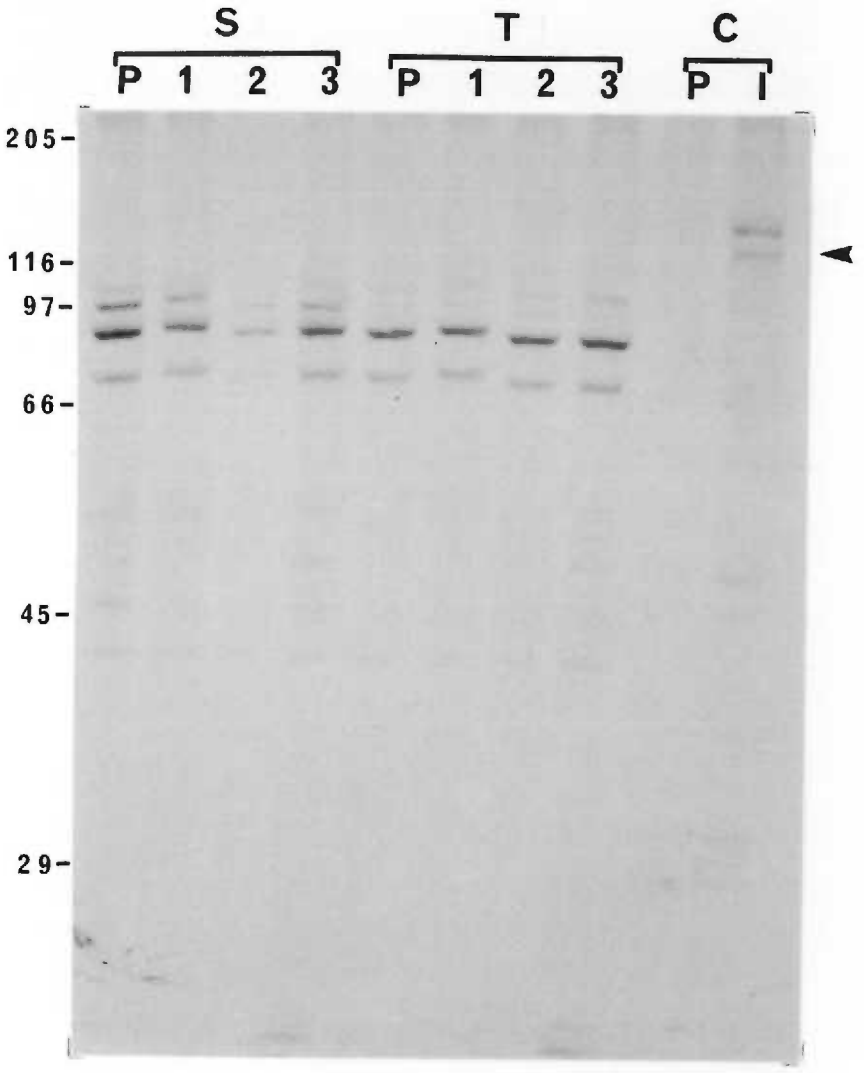


Figure 10. Western blot testing the antisera generated from recombinant vaccinia virus in promastigote (Prom.), amastigote (A) and Triton X-114 membrane lysates from promastigotes (T). Total protein lysates and Triton X-114 membrane lysates were prepared from promastigotes grown in tissue culture. Amastigote lysates were prepared from parasites purified from the nose lesions of infected guinea pigs. All the lysates were size fractionated in 10% SDS-PAGE and transferred to nitrocellulose filters. The nitrocellulose strips were reacted with pre-immune serum (P) and with antisera from three different bleedings from a rabbit infected with the recombinant virus (1, 2, 3). The molecular weight markers (kD) are indicated at the left.

Figure 11. Immunoprecipitation of extracts from ^{35}S-methionine labelled *L. enriettii* promastigotes using antisera raised against recombinant vaccinia virus. Immunoprecipitations were performed using pre-immune sera (P) or sera from three different bleedings (1, 2, 3) of a rabbit infected with the recombinant vaccinia virus. As a positive control (C) for immunoprecipitation, a human epithelial carcinoma cell line expressing the ANP receptor (courtesy of Dr. Michael Chinkers) was labelled *in vivo* with ^{35}S-methionine. Lysates from these cells were then immunoprecipitated with an immune serum (I) which reacts with the ANP receptor. This control immunoprecipitate produces two bands (arrowhead) with molecular weights above 116 kD, as expected for reaction with the ANP receptor (personal communication, Dr. Michael Chinkers). Antibody-antigen complexes were precipitated with protein A-Sepharose and then the immunoprecipitates were analyzed on 10% SDS-PAGE followed by the PPO enhancing procedure. *L. enriettii* promastigotes were lysed using either single detergent lysis buffer (S) or triple detergent lysis buffer (T) (36).


S
T
C
P 1 2 3 P 1 2 3 P I
205-
116-
97-
66-
45-
29-

these experiments failed to reveal the presence of antibodies directed against the Pro-1 polypeptide.

Epitope Tagging

In the epitope tagging experiments described here a small hydrophilic octapeptide called the Flag peptide (Asp Tyr Lys Asp Asp Asp Asp Lys) was engineered onto the C-terminus of Pro-1 by polymerase chain reaction (PCR) mutagenesis. This Flag system has been developed by the Immunex Corporation as an epitope tag for use in the purification and monitoring of proteins (28), and several monoclonal antibodies have been developed which recognize the Flag epitope. The advantage of epitope tagging is that it relies upon a commercially available monoclonal antibody circumventing the need to raise an antibody against a particular region of the Pro-1 protein. In this approach, it should be possible to separately tag both iso-1 and iso-2 and thus determine the location of each isoform. The disadvantage of this experiment is that modifying the protein with the epitope tag could cause mistargeting of the altered polypeptide.

In the experiments reported here, we generated a 600 bp PCR amplification product containing the C-terminus of the Pro-1 polypeptide fused to the Flag epitope, a hydrophilic octapeptide. This PCR product was then subcloned into the pANB87 plasmid, which is a clone containing the whole Pro-1 protein coding region in the *Leishmania* expression vector pALT-Neo (29). The resulting clone, designated pAN87-Flag, should express the epitope tagged Pro-1 protein upon transfection into *Leishmania* promastigotes. This plasmid was unintentionally transfected into *L. donovani* parasites, rather than *L. enriettii* , due to an apparent mislabelling of a parasite culture in our laboratory. In principal, these *L. donovani* transfectants should still be useful for localization of the Pro-1 polypeptide, since genes from one species of *Leishmania* can usually be functionally expressed in other

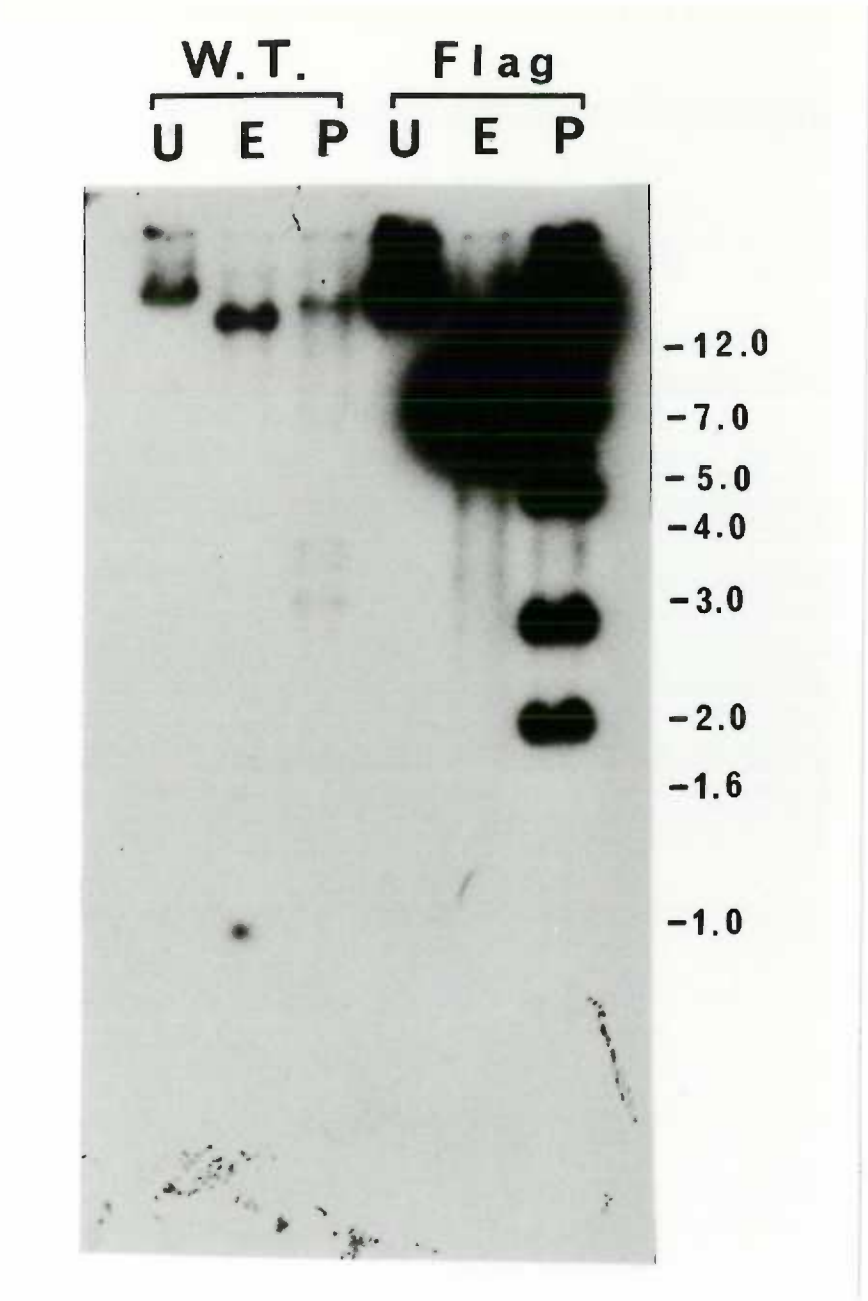


Figure 12. Southern blot analysis of the Pro-1 and epitope tagged Pro-1 gene in *L. donovani* . Uncut (U) and restriction enzyme-digested (E for *Eco* RI, P for *Pst* I) genomic DNA (2 μg) from wild type *L. donovani* (W.T.) and pAN87-Flag transfected *L. donovani* (Flag) were size separated on a 1% agarose gel, transferred onto nitrocellulose, and hybridized with the entire ^{32}P-labelled insert of Pro-1 protein coding region. The numbers at the right are the sizes (kb) of the molecular weight.

species of the parasite (42). A Southern blot of DNA from the transfected cells (Fig. 12) shows that the pAN87-Flag plasmid is present in the cell in a high copy number, approximately 10 times that of the Pro-1 gene which is present in about 7 to 9 copies (7). A Northern blot of RNA from cells transfected with the pAN87-Flag plasmid (Fig. 13) shows that pAN87-Flag RNA is expressed in these cells. In addition to the 3.3kb band which is the wild type Pro-1 RNA, there are several other bands of higher and lower molecular weight which are present only in the pAN87-Flag transfected cells and which represent transcripts from the pAN87-Flag plasmid. Genes which are expressed from the vector pALT-Neo frequently produce multiple transcripts, possibly due to the use of multiple polyadenylation sites, and / or spliced leader addition sites (42).

These pAN87-Flag transfected cells were tested by Western blot analysis to determine whether they were expressing the epitope tagged Pro-1 protein. In an initial Western blot (Fig. 14, panel A), probed with M2 monoclonal antibody directed against the Flag epitope, a band of about 50 kD was present in the lysate from pAN87-Flag transfected cells but not in the lysate from wild type cells. In addition, we performed a positive control containing a lysate from mammalian cells expressing the catalytic domain of the ANP receptor which had been modified with the Flag epitope (courtesy of Dr. Michael Chinkers). This positive control produced the expected band of 35 kD (Fig. 14, lane C for positive control) when probed with the M2 monoclonal antibody. These experiments suggested that the transfected cells expressed the Pro-1/Flag protein; the apparent molecular weight of 50 kD, rather than the predicted migration of 61 kD, could be due to the previously reported (14) tendency of very hydrophobic proteins, such as the mammalian glucose transporters, to migrate more rapidly than non-hydrophobic proteins of the same molecular weight.

However, subsequent Western blots using different lysates from wild type cells (Fig. 14, panel B) demonstrated that the 50 kD band was present in the non-transfected cells and

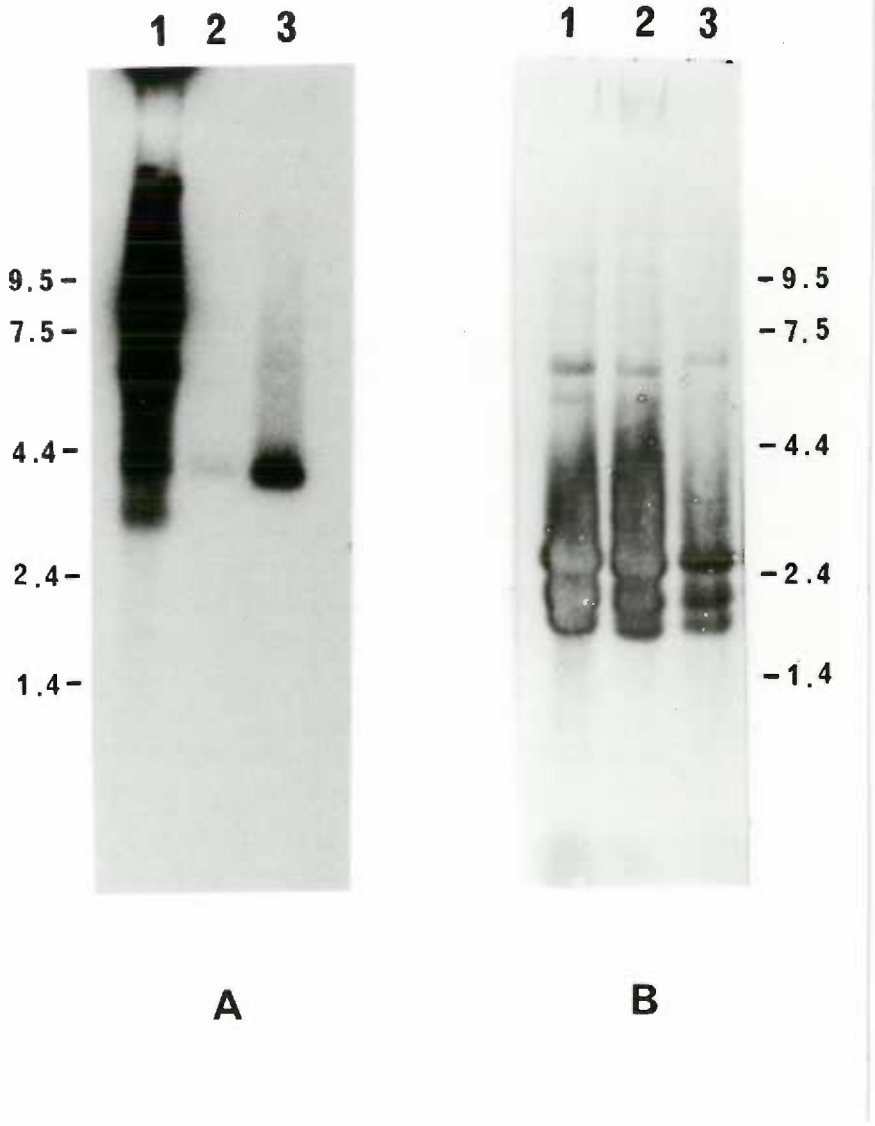
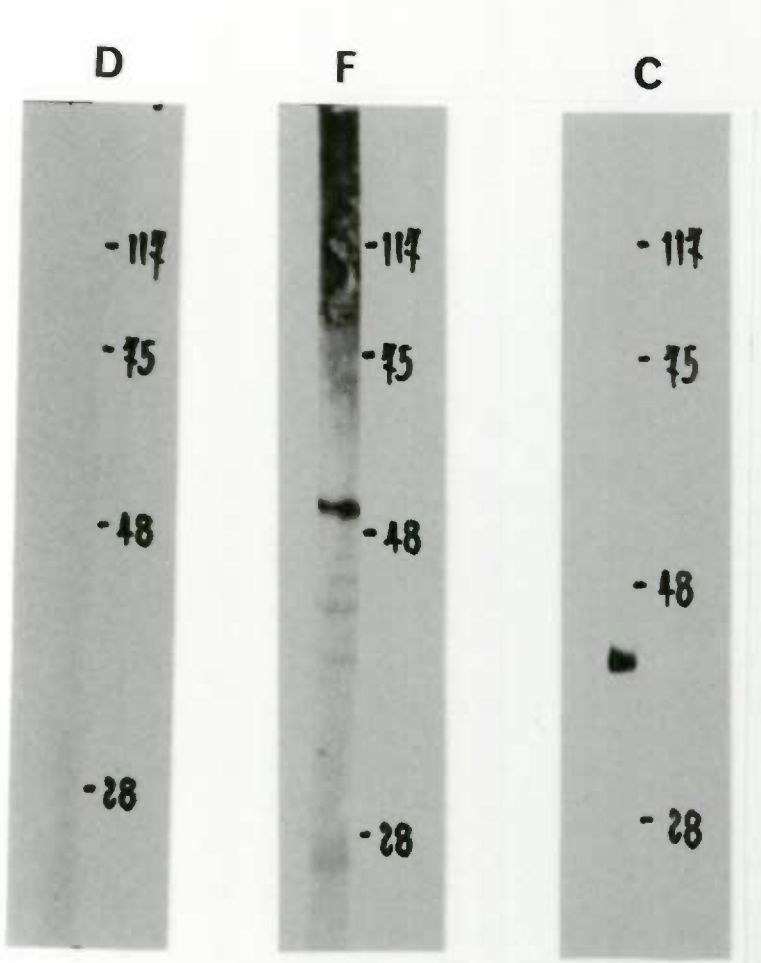
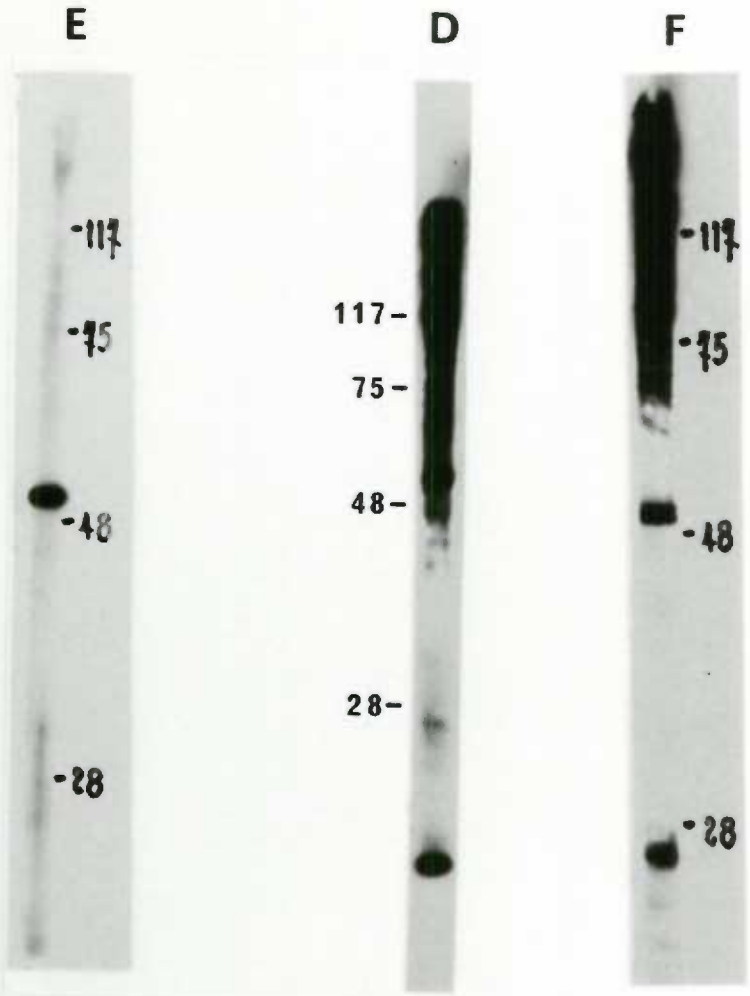


Figure 13. Expression of the Pro-1 gene and epitope tagged Pro-1 gene. Total RNA was isolated from pAN87-Flag transfected *L. donovani* (lane 1), pALT-Neo transfected *L. donovani* (lane 2) and wild type *L. donovani* (lane 3). Five μg total RNA were electrophoresed on 1% agarose-formaldehyde gel, transferred to nitrocellulose, stained with methylene blue (panel B) and then probed with the insert of the Pro-1 protein coding region (panel A).

Figure 14. Detection of epitope tagged Pro-1 and ANP receptor in Western blots.
Panel A: Lysates from wild type *L. donovani* (D) or pAN87-Flag transfected *L. donovani* (F), and control lysates (C) from mammalian cells expressing the catalytic domain of the ANP receptor which had been modified with the "Flag" epitope were separated in 10% SDS-PAGE and transferred to nitrocellulose filters. These filters were then probed with the anti-Flag monoclonal antibody M2. We subsequently determined that the lysates in strip D were degraded. Hence the experiment was repeated in panel B.
Panel B: Nitrocellulose strips of promastigotes of wild type *L. enriettii* (E), *L. donovani* (D) and pAN87-Flag transfected *L. donovani* were reacted with M2. Numbers are molecular weight markers (kD).



A



B

hence represents some cross-reacting material rather than the Flag modified Pro-1. Lysates from both *L. donovani* and *L. enriettii* produced a strongly cross-reacting band of about 50 kD and *L. donovani* lysates also produced a smear in the higher molecular weight range. Separation of the initial wild type lysate from Fig. 14A on a Laemmli gel followed by Coomassie Blue staining revealed that the proteins in this lysate had been degraded to small peptides (data not shown). Hence, the failure to detect the Flag cross-reacting material in the initial Western blot was due to the degradation of the wild type lysate. The observation that wild type *Leishmania* cross-react strongly with the Flag monoclonal antibody suggests that it will not be possible to use this antibody for immunolocalization studies.

Furthermore, it is not clear from these results whether the cells transfected with the pAN87-Flag plasmid actually expressed the epitope tagged Pro-1 protein, since the Western blots of wild type and transfected cells are essentially the same. It is possible that the RNA species present in the pAN87-Flag transfected cells (Fig. 13A, lane 1) are not functional in protein synthesis, due to their apparent difference in size (and hence structure) compared to the wild type Pro-1 mRNA (Fig. 13A, lane 3).

Immunofluorescence of wild type and pAN87-Flag transfected cells, using the M2 monoclonal antibody, demonstrates the problem posed by the presence of cross-reacting material in wild type cells. Both wild type and transfected cells (Fig. 15) produced the same fluorescent signal, distributed across the entire body of the cell.

Similar results were obtained from immunoprecipitation experiments using ^{35}S-methionine labelled wild type and transfected parasites. Immune precipitates using the M2 monoclonal antibody produced the same labelled bands in lysates from both wild type and transfected cells (Fig. 16).

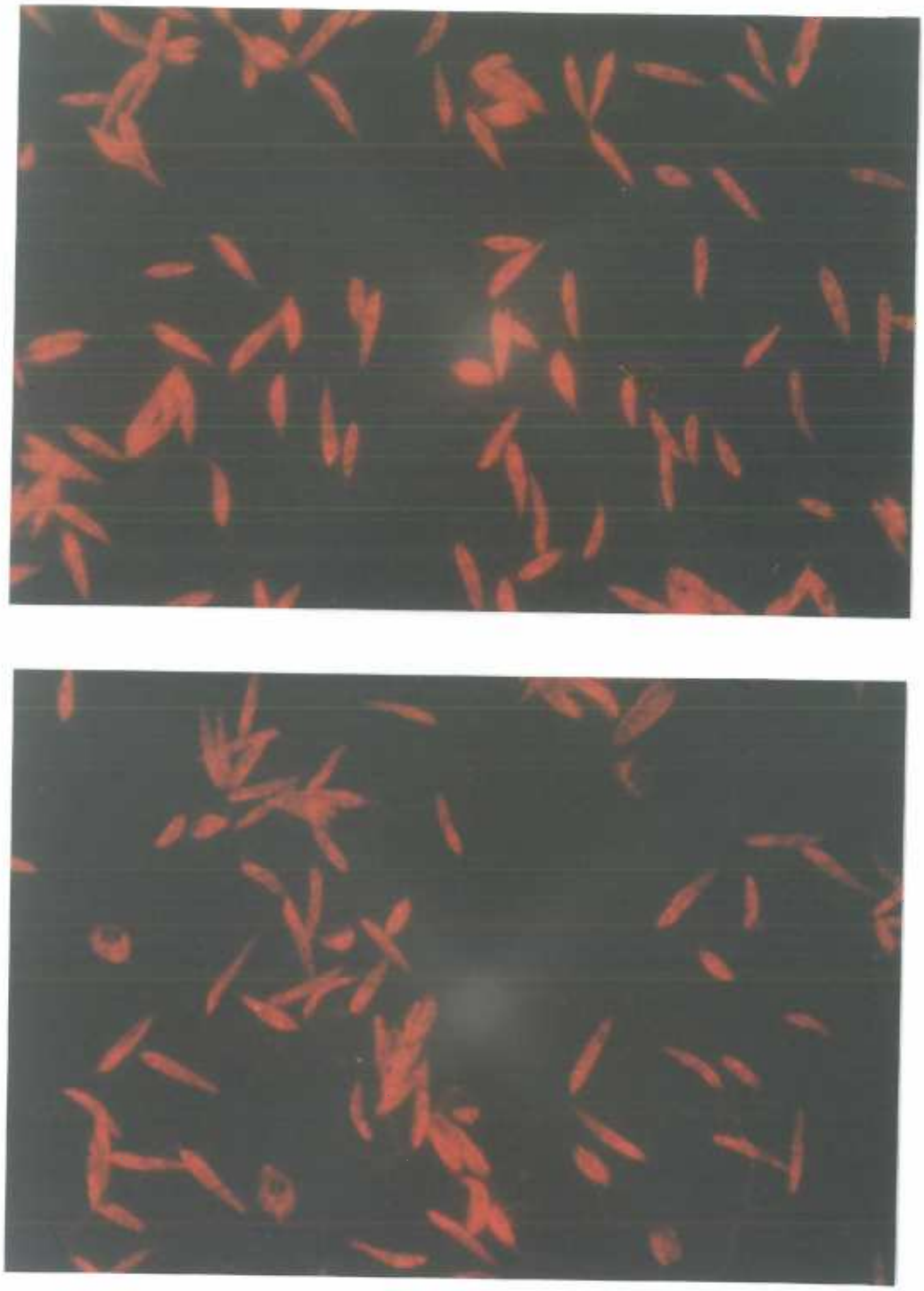


Figure 15. Indirect immunofluorescence using the anti-Flag M2 monoclonal antibody. Promastigotes of *L. donovani* transfected with either pALT-Neo (upper panel) or pAN87-Flag (lower panel) were fixed, incubated with the anti-Flag M2 monoclonal antibody, and then incubated with rhodamine-B conjugated goat anti-mouse IgG. Slides were examined under the fluorescence microscope at an excitation wavelength of 530-560 nm.

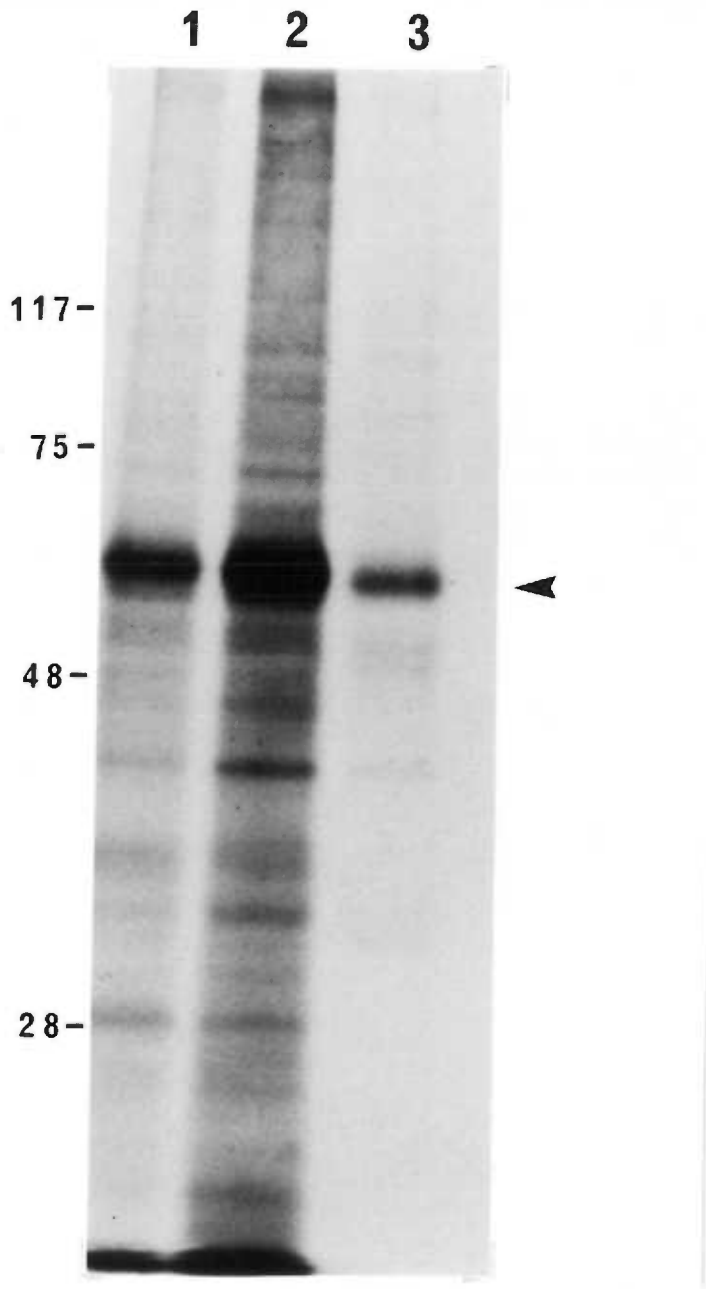


Figure 16. Immunoprecipitation of extracts from ^{35}S-methionine labelled *L. donovani* promastigotes transfected with either pALT-Neo (lane 1) or pAN87-Flag (lane 2) using the anti-Flag M2 monoclonal antibody. Land 3 is an immunoprecipitation of labelled wild type *L. enriettii* promastigotes using an anti α-tubulin monoclonal antibody.

Assessment of the 12CA5 Monoclonal Antibody for Use in Epitope Tagging

Since the Flag monoclonal antibody didn't work due to cross-reactivity, we wished to test other antibodies that could be used in epitope tagging. The 12CA5 monoclonal antibody recognizes a nine amino acid sequence (Tyr Pro Tyr Asp Tyr Pro Asp Tyr Ala) from the influenza heamaglutinin protein and has been used widely in epitope tagging experiments (22). We wished to determine whether or not this 12CA5 antibody could be used to detect epitope tagged proteins in *Leishmania* . It is very important to first test this antibody on wild type cells to see if it cross-reacts. Hence, we probed Westerns (Fig. 17) of *L. donovani* and *L. enriettii* lysates with 12CA5. The 12CA5 antibody does cross-react with lysates from both species, producing multiple bands. Hence this antibody will not be useful for epitope tagging, for the same reason that the M2 anti-Flag antibody did not work.

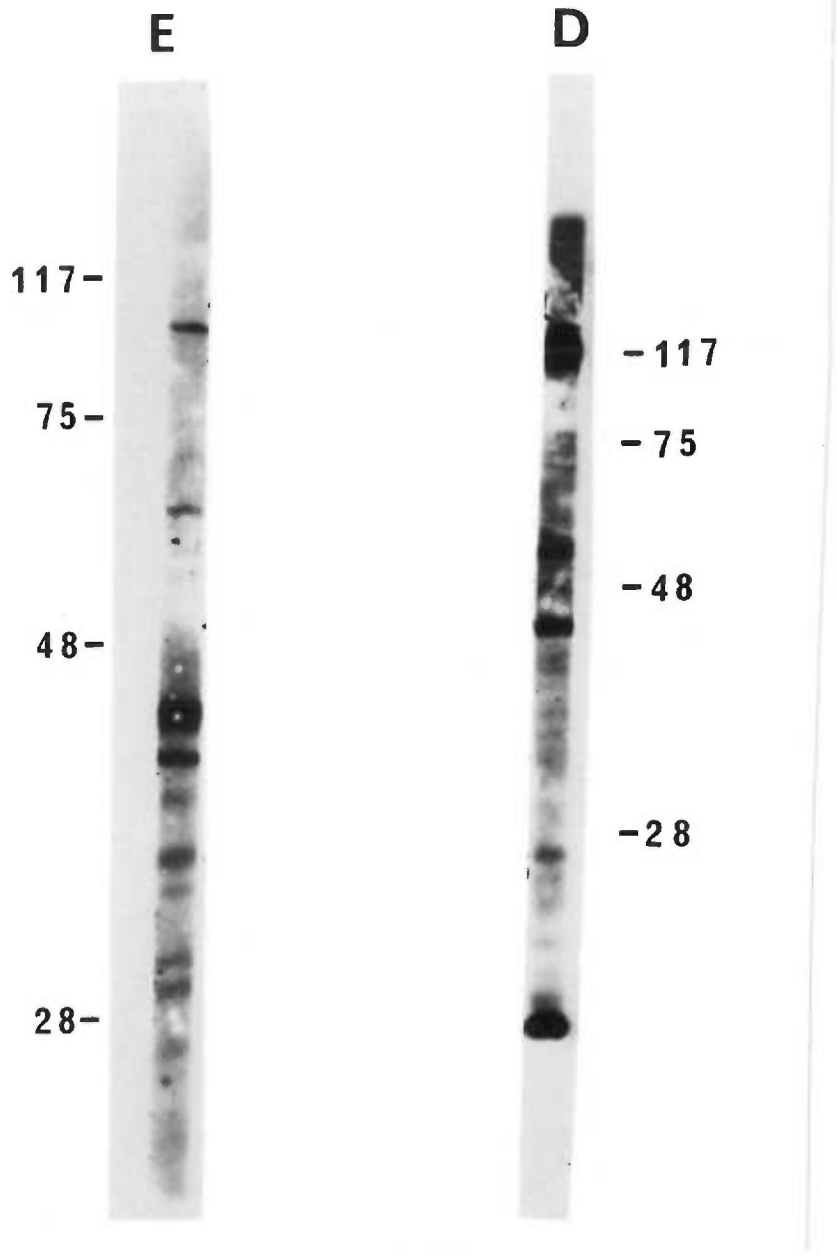


Figure 17. Western blot testing of 12CA5 monoclonal antibody against total protein lysates of *L. enriettii* (E) and *L. donovani* (D) promastigotes.

DISCUSSION

Role of transporters in the *Leishmania* life cycle

Leishmania species require two hosts to complete their life cycle, the sandfly and the mammalian macrophage. In both stages, *Leishmania* must acquire nutrients from the host. One striking example of this requirement for host-derived nutrients is the parasite's absolute dependence upon exogenous purines (6), which cannot be synthesized *de novo* (30). *Leishmania* promastigotes express two different nucleoside transporters (46) which efficiently salvage purines from the surrounding medium. In addition, the parasite relies upon its hosts to provide nutrients as a source of metabolic energy. When the parasite is in the sandfly, sugars are readily available and are utilized as a major source of energy (6). In contrast, in the macrophage lysosome sugars are not present at high concentrations, and fatty acids (presumably derived from macrophage degradation of serum lipoproteins) are the major metabolic energy source for the parasites (6). Consequently, the nutrient environment changes dramatically during the parasite life cycle. This may explain why some transporters like Pro-1 are expressed in a stage-specific manner.

In this thesis, we have studied a *Leishmania* protein which is a member of the glucose transporter superfamily. Transporters have been well studied at the molecular level in vertebrates, yeast and bacteria, but there is relatively little known about these proteins in parasitic protozoa. Transporters for glucose (47, 48), ribose (49), proline (48), folate (50, 51) and nucleosides (46, 52) have been characterized at the biochemical and/or genetic level in several *Leishmania* species. However, none of these genes encoding any of these transporters has been previously cloned, and essentially nothing is known concerning the structure or membrane location of these proteins. Studies on Pro-1 and related transporters will begin to fill this gap in knowledge.

Location of membrane proteins in Kinetoplastid protozoa

In *Leishmania* and the related parasite *Trypanosoma brucei* , there are several major membrane proteins which have been shown to be uniformly distributed over the plasma membrane: these include gp63, which is an ectoprotease and the major membrane protein of *Leishmania* (53); the variant surface glycoprotein (VSG) and the procyclic acid repetitive protein (PARP), the major membrane antigens of *T. brucei* (54, 55). These three proteins are anchored in the membrane *via* a glycosyl phosphatidylinositol (GPI) anchor, and the proteins themselves do not span the lipid bilayer. However, there are probably many integral membrane proteins that are widely distributed in the plasma membrane.

In contrast, several other membrane proteins in *T. brucei* have been shown to be localized to specific membrane domains. For example, the parasite transferrin receptor and the parasite LDL receptor are both localized to the flagellar pocket (20), which is a deep invagination of the plasma membrane located at the base of the flagellum. The flagellar pocket is believed to be the site of uptake of nutrients and macromolecules. In addition, a family of trypanosome receptor-adenylate cyclases were shown to be localized to the flagellar membrane (56, 57). Hence, some proteins are highly localized on the surface of the parasite.

Currently, little is known about *Leishmania* proteins in internal membranes. There is reason to suspect that some sugar transporters might be located on internal membranes. The glycosome is a membrane bound organelle which contains the glycolytic enzymes (19). To metabolize glucose, *Leishmania* parasites must transport this sugar across the plasma membrane and then across the glycosome membrane. Therefore, the parasite may need glucose transporters in both the plasma membrane and in the glycosome membrane.

Since Pro-1, a putative sugar transporter, has two isoforms which differ in only one domain, it is possible that one isoform is in the plasma membrane and the other in the glycosomal membrane.

Localization of mammalian glucose transporters

Among the mammalian facilitative glucose transporters, each isoform has a different sequence and tissue distribution (13). GLUT 1, which is the facilitative glucose carrier of human erythrocytes, is distributed over the plasma membrane of the red blood cells. GLUT 2 is preferentially expressed in the kidney and small intestine and might be responsible for the transport of glucose across the serosal surface of the epithelial cells which line the intestine and nephron. This isoform is located on the basolateral surface of these cells but not on the lumenal surface (13). GLUT 4, an insulin regulated glucose transporter expressed in insulin-responsive tissues, is localized predominantly in intracellular vesicles in the basal state. These membrane vesicles are translocated to the plasma membrane in response to insulin (13). Hence, different isoforms of mammalian glucose transporters can have dramatically different subcellular locations.

Strategy for localizing Pro-1 isoforms

For the mammalian glucose transporter GLUT 1, antibodies were raised against both N and C-terminal peptides (23, 24). The N-terminal antibody worked only on Western blots, while the C-terminal antibody worked on Westerns, immunoprecipitation, immunofluorescence and immnuoelectron microscopy. Hence, in initial experiments we attempted to raise an antibody against a C-terminal peptide of Pro-1. Since this antiserum did not produce any reactivity on Western blots, we turned to the following approaches.

Fusion proteins : The fusion proteins were made from the unique N-terminus and the first extracellular loop of Pro-1. The N-terminus is unique to iso-1, so an antibody directed against this region could be used to specifically localize iso-1. In addition, both regions are relatively large and hydrophilic, therefore they provide a better chance to obtain an antibody than if a shorter or less hydrophilic regions were used. Furthermore, these regions contain convenient restriction sites for subcloning into expression vectors.

Vaccinia virus : Recombinant vaccinia virus containing the Pro-1 gene should be able to express the entire Pro-1 transporter. This method of raising antibodies is a different route of immunization than the antibody obtained from fusion proteins, since it involves infection of animals with a live virus which expresses the protein of interest in the infected cells. This approach has been used successfully to generate antibodies against some membrane spanning proteins, such as the atrial natriuretic peptide (ANP) receptor (27)

Epitope tagging : The epitope tagging approach has some potential advantages over the use of antibodies generated directly against the protein of interest. Tagging a protein with a small peptide epitope allows the surveillance of the protein with a well-characterized monoclonal antibody. This approach does not depend upon the ability to raise an antibody against Pro-1 itself. The C-terminus of Pro-1 was chosen as the tagging site, since the antibodies against the C-terminus of mammalian transporters were used successfully in immunofluorescence and immunoelectron microscopy. Therefore, the C-terminus of the native transporters is likely to be accessible to the antibodies. Furthermore, the ends of proteins may be more likely to be susceptible to modification without affecting function. Recently, epitope tagging on the C-terminus has been used successfully to modify and localize the mammalian GLUT 4 transporter (Dr. Rob Piper, personal communication).

Evaluation of antisera directed against Pro-1

None of the antisera tested here showed any convincing reactivity with Pro-1 on Western blots or by immunoprecipitation. It is possible that these fusion proteins might be non-immunogenic for some unknown reasons. However, it is worth considering some of the potential reasons why these approaches may have failed and to contemplate alternative approaches to raise anti-Pro-1 antibodies.

N-terminal fusion protein (N1, N2)

One difficulty encountered with antibodies directed against the N-terminus of mammalian GLUT 1 was their ineffectiveness in any test except Western blots (23, 24). Consequently, this region of glucose transporters may not be accessible to antibodies when these proteins are in their native state. However, an antibody against the N-terminus of iso-1 could still be useful for localization of this isoform, even if it was only reactive on Western blots. Since glycosomes can be fractionated from plasma membranes using equilibrium sucrose gradients (58, 59), it would be possible to test glycosomal and plasma membrane fractions on Western blots for the presence or absence of iso-1.

There are several potential reasons for the failure to obtain antibodies that react with the N-terminus of iso-1. It is possible that the N-terminus of iso-1 is not hydrophilic enough. This region has a lower hydrophilicity than the C-terminus, first hydrophilic loop, or N-terminus of iso-2, as determined by Kyte and Doolittle plots (60) of this sequence. Alternatively, to generate a useful antibody, it may be necessary to use a larger region of the N-terminal tail than the 67 amino acid segment present in the N1 and N2 fusion proteins. In particular, it may be important to include the N-terminal amino acids of this tail in the fusion protein, so that antibodies which are directed against the amino terminal

sequence of iso-1 are elicited. In addition, this amino terminal peptide may need to be placed on the N-terminus of the fusion protein, so that the antibody is raised against this peptide in the correct structural context. One approach would be to amplify the entire N-terminal tail of iso-1 by PCR and to clone this PCR product into an expression vector which would place the peptide on the N-terminus of the fusion protein.

Fusion protein containing the hydrophilic loop (L1)

The high cysteine content (seven cysteines) of the first extracellular loop may indicate the presence of extensive disulfide bonds in this region of the native Pro-1 protein. If this is the case, the conformations presented to the immune system may not be like those present in either the native or denatured Pro-1. Consequently, these antibodies might not be able to recognize Pro-1 on either Western blots or on immunoprecipitation.

Vaccinia Virus

It is not clear why no antibody was obtained against the Pro-1 protein expressed by the recombinant vaccinia virus. Although the Pro-1 RNA was produced by the recombinant virus (Fig. 9, lane 2), it is not known whether stable protein was made in these infected cells. Hence, one possible explanation for the failure of this virus to elicit antibodies is that the infection did not result in sufficient production of Pro-1 protein in the infected animal.

Epitope tagging

Monoclonal antibodies against the 'Flag' and influenza epitopes both cross-reacted strongly with wild type *Leishmania* , hence they will not be useful for immunolocalization of Pro-1.

Although epitope tagging is still a viable approach, it will be essential to obtain an antibody lacking strong cross-reactivity with the parasite. We have obtained several anti-peptide antibodies directed against C-terminal peptides of GLUT 1, GLUT 2 and GLUT 4 (gift of Dr. Rob Piper). These antisera have been tested on Western blots of *L. enriettii* and *L. donovani* lysates for cross-reactivity. While the anti-GLUT 1 and GLUT 4 antisera do cross-react strongly, the anti-GLUT 2 antiserum does not. In future experiments, the appropriate peptide (Thr Val Gln Met Glu Phe Leu Gly Ser Ser Gly Thr Val) will be used to modify the C-terminus of each isoform of Pro-1.

Potential of a fusion protein containing the C-terminal tail of Pro-1

The failure of the C-terminal peptide of Pro-1 to elicit antibodies originally directed us to other regions of the Pro-1 protein. However, the problem may have been the method of coupling the peptide to KLH, rather than inherent non-immunogenicity of the C-terminus. GLUT 4 peptides coupled to KLH by glutaraldehyde did not elicit antibodies (personal communication, Dr. Rob Piper). However, when they were coupled to KLH at a single site *via* a N-terminal cysteine residue, they were able to produce antibody.

In addition, a glutathione S-transferase fusion protein containing the C-terminal tail of another related *Leishmania* transporter, designated D1 (45), did produce a useful antiserum (Chris Langford, unpublished results). These results encouraged us to try a similar approach with Pro-1.

The C-terminal tail from His 536 to Glu 567 (7) of Pro-1(Fig. 3) was amplified by PCR and cloned into pGEX-2T. A 29 kD fusion protein (designated **fusion protein C1,** where C indicates that the sequence originates from the carboxyl terminus of Pro-1) was

generated. This fusion protein was purified, emulsified in Freund's Complete Adjuvant and injected into rabbits. Currently, we are awaiting the results of bleeds.

This antibody, if successful, would not distinguish between iso-1 and iso-2 directly, but would allow assessment of total Pro-1 distribution. Furthermore, it would be possible to delete the iso-1 gene by targeted gene disruption (61) and then determine whether antibody staining disappears from some discrete region (e.g. glycosomes) in these deletion mutants.

Summary

In this thesis, I have attempted to raise antibodies against both isoforms of the Pro-1 protein with the ultimate intention of determining the subcellular location of each isoform. Although I have used several approaches, none of the antisera obtained to date react with the Pro-1 protein. These negative results reflect the difficulty frequently encountered in raising antisera against very hydrophobic multi-membrane spanning proteins.

However, these experiments have at least ruled out several potential approaches to the problem and have suggested alternative strategies which may generate useful antibodies.

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October 28, 1992

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