Localization and Targeting of an Unusual Pyridine Nucleotide Transhydrogenase in *Entamoeba histolytica*

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Pyridine nucleotide transhydrogenase (PNT) catalyzes the direct transfer of a hydride-ion equivalent between NAD(H) and NADP(H) in bacteria and the mitochondria of eukaryotes. PNT was previously postulated to be localized to the highly divergent mitochondrion-related organelle, the mitosome, in the anaerobic/microaerophilic protozoan parasite *Entamoeba histolytica* based on the potential mitochondrion-targeting signal. However, our previous proteomic study of isolated phagosomes suggested that PNT is localized to organelles other than mitosomes. An immunofluorescence assay using anti-*E. histolytica* PNT (ErPNT) antibody raised against the NAD(H)-binding domain showed a distribution to the membrane of numerous vesicles/vacuoles, including lysosomes and phagosomes. The domain(s) required for the trafficking of PNT to vesicles/vacuoles was examined by using amoeba transformants expressing a series of carboxyl-terminally truncated PNTs fused with green fluorescent protein or a hemagglutinin tag. All truncated PNTs failed to reach vesicles/vacuoles and were retained in the endoplasmic reticulum. These data indicate that the putative targeting signal is not sufficient for the trafficking of PNT to the vesicular/vacuolar compartments and that full-length PNT is necessary for correct transport. PNT displayed a smear of >120 kDa on SDS-PAGE gels. PNGase F and tunicamycin treatment, chemical degradation of carbohydrates, and heat treatment of PNT suggested that the apparent aberrant mobility of PNT is likely attributable to its hydrophobic nature. PNT that is compartmentalized to the acidic compartments is unprecedented in eukaryotes and may possess a unique physiological role in *E. histolytica*.

Pyridine nucleotide transhydrogenase (PNT) participates in the bioenergetic processes of the cell. PNT generally resides on the cytoplasmic membranes of bacteria and the inner membrane of mammalian mitochondria (3, 16) and utilizes the electrochemical proton gradient across the membrane to drive the pyridine nucleotide transhydrogenase reaction

\[ \text{NADP}^+ + \text{H}^+ \rightarrow \text{NAD}^+ + \text{H}_2 \text{O} \]

out

H

in

However, our previous study of isolated phagosomes suggested that PNT is localized to organelles other than mitosomes. An immunofluorescence assay using anti-*E. histolytica* PNT (ErPNT) antibody raised against the NAD(H)-binding domain showed a distribution to the membrane of numerous vesicles/vacuoles, including lysosomes and phagosomes. The domain(s) required for the trafficking of PNT to vesicles/vacuoles was examined by using amoeba transformants expressing a series of carboxyl-terminally truncated PNTs fused with green fluorescent protein or a hemagglutinin tag. All truncated PNTs failed to reach vesicles/vacuoles and were retained in the endoplasmic reticulum. These data indicate that the putative targeting signal is not sufficient for the trafficking of PNT to the vesicular/vacuolar compartments and that full-length PNT is necessary for correct transport. PNT displayed a smear of >120 kDa on SDS-PAGE gels. PNGase F and tunicamycin treatment, chemical degradation of carbohydrates, and heat treatment of PNT suggested that the apparent aberrant mobility of PNT is likely attributable to its hydrophobic nature. PNT that is compartmentalized to the acidic compartments is unprecedented in eukaryotes and may possess a unique physiological role in *E. histolytica*.
Chinese hamster ovary (CHO) cells were maintained in F12 medium (Invitrogen, San Diego, CA) supplied with 10% fetal calf serum (Medical Biological Laboratory, Woburn, MA) at 37°C with 5% CO₂. Escherichia coli strains DH5α and BL21(DE3) were purchased from Life Technologies (Tokyo, Japan) and Novagen (Madison, WI), respectively. LysoTracker Red DND-99 and Cell-Tracker Orange CMTRMR [5-(and-6)-[[4-chloromethyl]benzoyl]amino]tetramethylrhodamine] were purchased from Molecular Probes (Eugene, OR). All other chemicals of analytical grade were purchased from Sigma-Aldrich unless otherwise stated.

**Plasmid construction.** Standard techniques were used for routine DNA manipulation, subcloning, and plasmid construction as previously described (38). To produce E. coli recombinant proteins, a coding region corresponding to dIIb (amino acids [aa] 565 to 960) of EhPNT (EhPNTdI) was amplified from an E. histolytica cDNA library by using a pair of appropriate primers designed on the basis of the nucleotide sequences in the Genbank database (accession number L39933) (8), with BamHI and XhoI restriction enzyme sites. The sense and antisense primers were 5'-GGACATATGCTTCTTCTTCAATACTAGAACTTTGTGCTCAT-3' and 5'-GGATCC ATTCTTCATTTAATTCTTCAAATCCTTTC-3', respectively, where boldface type indicates the BamHI or XhoI site. The PCR products were cloned into the BamH I- and XhoI-digested vector pET47b (Novagen, Madison, WI), and the resulting plasmid was designated pHisPNTdI. To generate vectors to express either full-length or truncated forms of EhPNT fused to HA in the amoeba, a protein-coding region corresponding to full-length EhPNT (Genbank accession number AAC41577) (aa 1 to 1083), the 14-aa amino-acid residues of asparagine-linked glycoproteins, according to the manufacturer's instructions.

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**Antibiotics.** Anti-EhPNT antibody was raised against purified EhPNTdI protein in rabbits commercially (Operon, Tokyo, Japan). Anti-HA 110 mouse monoclonal antibody was purchased from Berkeley Antibody (Berkeley, CA). Anti-EhSec61 α-subunit and anti-E. histolytica dolichol-P-mannose synthase (EAD-PMS) antibodies were a gift from Rosana Sanchez-Lopez (37). Anti-galactosidase N-acetylglucosamyltransferable lectin (H. influenzae) was obtained by Dr. Michael A. Petri, Jr. Alexa Fluor 488- or 568-conjugated anti-mouse and anti-rabbit IgGs were purchased from Invitrogen. Alkaline phosphatase-conjugated goat anti-rabbit and goat anti-mouse IgGs were bought from Jackson ImmunoResearch Laboratories (Bar Harbor, ME). Anti-GFP rabbit antibody was purchased from Medical Biological Laboratory International.

**Immunoprecipitation.** Approximately 3 x 10⁶ cells of EhPNT-HA- and Cnp60-HA-expressing amoebae were lysed in lysis buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1% Triton X-100, and 0.5 mM EGTA) (34). The soluble lysate, after centrifugation at 15,000 x g, was incubated with protein G-Sepharose beads (30 μl of a 50% slurry) (Amersham Biosciences, Uppsala, Sweden) premixed with anti-HA antibody (0.5 μg) or anti-HA and anti-Hgl antibodies, respectively.

**Immunoblot analysis.** Whole-cell lysate and immunoprecipitated samples were separated on either a 12% or 15% (wt/vol) SDS-polyacrylamide gel and subsequently electrotransferred onto nitrocellulose membranes (Hybond-C Extra; Amersham Biosciences, Little Chalfont, Bucks, United Kingdom) as described previously (41). The membranes were blocked by incubation in 5% nonfat dried milk in TBST (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, and 0.05% Tween 20) for 1.5 h at room temperature. The blots were reacted with primary anti-EhPNT rabbit or anti-HA mouse antibody at a dilution of 1:500 to 1:1,000. The membranes were washed with TBST and further reacted with alkaline phosphatase-conjugated anti-rabbit or anti-mouse IgG antibody (1:1,000) room temperature for 1.5 h. After further washing with TBST, specific proteins were visualized with an alkaline phosphatase conjugate substrate kit (Bio-Rad, Hercules, CA).

**Deglycosylation of PNT.** The EhPNT-HA-expressing transformant was cultured with 3 μg/ml of tunicamycin (Sigma, St. Louis, MO) for 24 h to inhibit asparagine-linked glycosylation according to a protocol described previously (22). For the chemical deglycosylation of EhPNT, EhPNT and fetuin were dried in a Speed Vac. Ice-cold trifluoromethanesulfonic acid (TFMS) (73:10, vol/vol) containing 0.1% SDS to the mixture. Anisol was extracted three times with 250 μl ethyl ether. Diallyl was performed against 2 mM pyridine acetate buffer. After dialysis, the samples were subjected to SDS-PAGE and silver staining. Immunoprecipitated EhPNT was also digested with PNGase F (New England Biolabs, Ipswich, MA), an amylase that cleaves between the innermost N-acetylgalactosamine and asparagine residues of asparagine-linked glycopeptides, according to the manufacturer's instructions.

**Immunofluorescence assay and organellar staining.** Amoeba transformant or wild-type amoebae in a logarithmic growth phase were harvested, transferred into 8-mm round wells on glass slides, and incubated for 30 min at 35°C to let trophozoites attach to the glass surface. An indirect immunofluorescence assay was performed as previously described (36). Briefly, amoebae were fixed with 3.7% paraformaldehyde in PBS for 10 min at room temperature. The cells were then permeabilized with 0.05% Triton X-100 in PBS for 5 min. The samples were reacted with anti-EhPNT (1:100), anti-EhSec61 α-subunit (1:30), anti-EhPDPM antibody (1:30), anti-GFP (1:1,000) rabbit antibody, anti-HA (1:1,000) mouse antibody, or preimmune rabbit serum (1:100). The samples were then reacted with Alexa Fluor 488- or 568-conjugated anti-mouse or anti-rabbit IgG (1:1,000). For the staining of endosomes or late endosomes/lysosomes, amoebae were incubated with BI-S-33 medium containing 2 mg/ml rhodamine isothiocyanate (RITC)-dextran for 10 to 60 min (for endosomes) or LysoTracker Red DND-99 (Molecular Probes, Eugene, OR) (1:500) for 12 h (for late endosomes/lysosomes), respectively. To visualize phagosomes, CHO cells prestained with 10 μM CellTracker Orange or 20 μM CellTracker Blue were added to E. histolytica trophozoites in 8-mm wells on a glass slide and incubated for 10 to 60 min. The samples were examined on an LSM 510 Meta confocal laser scanning microscope (Carl Zeiss, Thornwood, NY). Images were further analyzed by using LSM510 software.

**RESULTS**

**Identification of two isotypes of PNT in E. histolytica.** Two isozymes of PNT have been identified in the E. histolytica genome database at Pathema (http://pathema.tigr.org/tigr-scripts/pathema/) (EHI_055400 and EHI_040403), corresponding to GenBank accession numbers XP_001914099 and AAC41577, respectively. They showed 90% mutual amino acid identity. The latter EhPNT isotype (EHI_040403) is 1,083 aa long with a predicted molecular mass of 117.0 kDa and a pI of 5.39. This predicted protein is identical to the PNT protein previously reported (GenBank accession number AAC41577).
FIG. 1. Expression of PNT in *E. histolytica*. (A) Immunoblot analysis of native EhPNT. Approximately 10 µg of total lysate was electrophoresed on a 12% SDS-polyacrylamide gel and subjected to an immunoblot assay with anti-EhPNT antibody or preimmune serum. M, molecular mass marker. (B) Immunoprecipitation of EhPNT. The lysates derived from the transformant expressing either EhPNT-HA (“PNT-HA”) or EhCPn60-HA (“CPN60-HA”) and the wild-type strain (“WT”) were subjected to immunoprecipitation with anti-HA antibody, followed by immunoblot analysis with anti-HA antibody. Lysate derived from the wild-type amoebae was also used directly for immunoblot analysis as a control. An arrowhead and an arrow indicate heavy and light chains of anti-HA antibody, respectively. IP, immunoprecipitation; IB, immunoblot. (C) Effect of heat treatment on the mobility of EhPNT. The lysates derived from the transformant expressing either anti-EhPNT antibody or preimmune serum were subjected to heat treatment on an SDS-PAGE gel. Approximately 10 µg of total lysate was electrophoresed on a 12% SDS-polyacrylamide gel and subjected to an immunoblot assay with anti-EhPNT antibody. M, molecular mass marker.

FIG. 2. Deglycosylation of EhPNT. (A and B) Deglycosylation by TFMS or PNGase F. The lysates obtained from the transformant expressing EhPNT-HA were subjected to immunoprecipitation (IP) with anti-HA antibody, followed by TFMS or PNGase F treatment and immunoblot detection with anti-HA antibody (right). Fetuin was used as a control, and gels were stained with silver (A) or Coomassie brilliant blue (B) (left). Filled arrowheads indicate untreated fetuin. Arrows indicate fetuin deglycosylated by TFMS (A) or PNGase F (B). Open arrowheads indicate EhPNT. (C) Deglycosylation by tunicamycin. Lysates from the transformant expressing EhPNT-HA cultured with tunicamycin were subjected to immunoprecipitation with either anti-EhHgl or anti-HA antibody, followed by immunoblot analysis with either anti-EhHgl or anti-HA antibody, respectively. A filled arrowhead or an arrow indicates untreated or deglycosylated EhHgl, respectively. An open arrowhead indicates EhPNT.

Expression of PNT in *E. histolytica* trophozoites. Immunoblot analysis of the trophozoite lysate using anti-EhPNT antibody showed a smear of >120 kDa (Fig. 1A). The size of the smear was unexpected because the predicted molecular masses of EhI_055400 and EhI_014030 were 119.0 and 117.0 kDa, respectively. To verify that this was not due to the cross-reactivity of anti-EhPNT antibody, we immunoprecipitated EhPNT from the EhPNT-HA-expressing transformant with anti-HA antibody, followed by immunoblotting with anti-HA (Fig. 1B) or anti-EhPNT (data not shown) antibody. Immunoprecipitated EhPNT-HA was also recognized as a smear of >120 kDa, similar to that of endogenous EhPNT.

To examine whether the smear was due to posttranslational modifications such as glycosylation, we treated immunoprecipitated EhPNT-HA with TFMS or PNGase F. The pattern of immunoblots with anti-HA antibody was not affected, while the apparent molecular mass of control fetuin decreased (Fig. 2A and B). In addition, the treatment of the trophozoites with tunicamycin did not affect the mobility of EhPNT, while the mobility of control Hgl increased (Fig. 2C). These data are consistent with the notion that EhPNT is not glycosylated. We next examined whether aberrant mobility is due to unusual tertiary structures. We compared the patterns of the amoebic lysates, mixed with a one-third volume of 4× SDS-PAGE sample buffer (0.25 M Tris-HCl [pH 6.8], 8% SDS, and 8% 2-mercaptoethanol) and either incubated at 95°C for 5 min or left unheated, on SDS-PAGE gels. When the sample was electrophoresed without heating, EhPNT was observed as a polypeptide of the predicted size (Fig. 1C). The exclusion of 2-mercaptoethanol did not affect mobility (data not shown). A similar observation was previously reported for membrane proteins, including serotonin transporter (24) and severe acute respiratory syndrome (SARS)-associated coronavirus mem-
brane protein (18). In the latter case, three hydrophobic regions of 10 to 35 aa were shown to be responsible for the heat-induced aggregation of the membrane protein (18), suggesting that the heat-induced change of mobility of \( \text{EhPNT} \) on SDS-PAGE is likely due to the hydrophobic nature of the protein.

**Subcellular distribution of \( \text{EhPNT} \).** An immunofluorescence assay using anti-\( \text{EhPNT} \) antibody showed that \( \text{EhPNT} \) is associated with the membranes of vesicles and vacuoles varying in size, or sometimes dot-like structures, scattered throughout the cytosol (Fig. 3A). We also examined the intracellular distribution of \( \text{EhPNT} \) using the amoebic transformant that expressed \( \text{EhPNT} \) (EH1_014030) with the carboxyl-terminal HA tag. The pattern of exogenous \( \text{EhPNT-HA} \) (EH1_014030) and that of endogenous \( \text{EhPNT} \) (a sum of EH1_055400 and EH1_014030) were indistinguishable (Fig. 3B). Since \( \text{EhPNT} \) was previously postulated to be localized to mitosomes (8), we next examined the localization of \( \text{EhPNT} \) and \( \text{EhCpn60} \), the authentic marker of mitosomes, in the amoebic transformant expressing \( \text{EhCpn60-HA} \) (26) using anti-\( \text{EhPNT} \) and anti-HA antibodies. No colocalization of \( \text{EhPNT} \) and \( \text{EhCpn60} \) was observed (Fig. 4).

Since \( \text{EhPNT} \) was previously detected in isolated phagosomes (32, 33), we examined the localization of \( \text{EhPNT} \) during the phagocytosis of CHO cells. Wild-type amoebae were incubated with CellTracker Orange-loaded CHO cells for 10 to 60 min to allow the ingestion of CHO cells. An immunofluorescence assay using anti-\( \text{EhPNT} \) antibody (Fig. 5A) showed that the phagocytosed CHO cells were associated with \( \text{EhPNT} \) at all time points (10, 20, and 60 min; only the images at 60 min are shown). The percentage of association gradually increased during the course of phagocytosis (65% ± 6%, 79% ± 9%, and 80% ± 8% at 10, 30, and 60 min, respectively). We then examined whether \( \text{EhPNT} \) is localized to lysosomes using LysoTracker Red, a membrane-diffusible probe accumulated in acidic organelles (5). We found that the LysoTracker-labeled acidic compartment, the size and number of which were consistent with previous findings (28, 36), was associated with \( \text{EhPNT} \) under steady-state conditions (79% ± 6% association) (Fig. 5B). To see whether \( \text{EhPNT} \) is also associated with endosomes, we examined the localization of an endocytosed...
fluid-phage marker, RITC-dextran. EhPNT was only partially associated with RITC-dextran-containing endosomes, which were observed as tiny dot-like structures or a multivesicular body as previously shown (29), at each time point (10, 30, or 60 min; only the images at 60 min are shown) (Fig. 5C). We also examined whether EhPNT is associated with mitosomes during the phagocytosis of CHO cells. An immunofluorescence assay using the amoebic transformant expressing EhCpn60-HA, CellTracker Blue-loaded CHO cells, anti-EhPNT, and anti-HA antibody showed no colocalization of mitosomes and EhPNT (Fig. 5D).

All domains are essential for the vesicular/vacuolar distribution of EhPNT. To define the domain necessary for vesicular/vacuolar targeting of EhPNT, we created amoeba transformants expressing the HA-tagged or GFP-fused protein containing various domains of PNT (Fig. 6A). The amoebic transformant expressing GFP fused with the 14-aa amino-terminal putative TS showed a cytoplasmic distribution (Fig. 6B), although the sequence MSTSSSIIEEYVFNY appeared to contain the elements implicated for the TS (rich in hydroxylated and hydrophobic amino acids). The transformants expressing TS/H11001dIIb-HA, TS/H11001dIIb/H11001dIII-HA, TS/H11001dIIb/H11001dIII/L-HA, or TS/H11001dIIb/H11001dIII/L/H11001dI-HA showed a distribution that overlapped that of the endoplasmic reticulum (ER), visualized with anti-EhSec61/H9251-subunit antibody (27). The ER pattern was also confirmed with anti-EhDPMS antibody (Fig. 6B). Full-length EhPNT-HA did not overlap the ER visualized with either anti-EhSec61 α-subunit or anti-EhDPMS antibody. Fractionation of the amoeba lysate followed by immunoblot analysis with anti-HA or anti-GFP an-
A

**Targeting Signal (TS)-GFP**

| Expression          | HA Epitope |
|---------------------|------------|
| TS + dllb-HA        | TS dllb HA |
| TS + dllb + dlll-HA | TS dllb dlll HA |
| TS + dllb + dlll + Linker-HA | TS dllb dlll Linker dl HA |
| TS + dllb + dlll + Linker + dl-HA | TS dllb dlll Linker dl dlIa HA |
| Full length PNT-HA  | TS dllb dlll |

B

**Anti-GFP**

| Expression          | Anti-GFP | Anti-HA | Merge | Phase |
|---------------------|----------|---------|-------|-------|
| EhTS-GFP            | Anti-EhSec61 | Anti-HA | Merge | Phase |
| EhTS + dllb-HA      | Anti-EhDPMS | Anti-HA | Merge | Phase |
| EhTS + dllb + dlll-HA | Anti-EhSec61 | Anti-HA | Merge | Phase |
| EhTS + dllb + dlll + L-HA | Anti-EhSec61 | Anti-HA | Merge | Phase |
| Full length EhPNT-HA | Anti-EhSec61 | Anti-HA | Merge | Phase |

**FIG. 6.** Localization of a series of truncated *EhPNT* proteins. (A) Schematic representation of HA epitope-tagged or GFP-fused recombinant *EhPNT* used in the study. Domains, epitope (or GFP), and amino acid numbers are shown. (B) Localization of epitope-tagged or GFP-fused carboxyl-terminally truncated *EhPNT* recombinant proteins. The transformants expressing *EhTS-GFP*, *EhTS + dllb-HA*, *EhTS + dllb + dlll-HA*, *EhTS + dllb + dlll + L-HA*, and *EhTS + dllb + dlll + L + dl-HA* and *EhPNT-HA* were subjected to an immunofluorescence assay using anti-HA (or anti-GFP for *EhTS-GFP*) and anti-*EhSec61* α-subunit (or anti-*EhDPMS* for the *EhPNT-HA* transformant) antibodies. Bar, 10 μm.

Mitochondria showed that all truncated forms of *EhPNT* except for TS-GFP were partitioned into the 5,000 × g and 100,000 × g pellet fractions, while TS-GFP was fractionated into the 100,000 × g supernatant fraction (data not shown).

**DISCUSSION**

Mitosomes have been identified in several parasitic protozoan lineages such as *E. histolytica*, *Giardia intestinalis*, *Trichomonas hominis*, and *Cryptosporidium parvum* (21, 34, 42, 43, 50). *E. histolytica* was previously considered to be an early-branching “amitochondriate,” as it lacks conventional mitochondria as well as other organelles typically found in most eukaryotes, such as peroxisomes, the rough ER, and the Golgi apparatus. However, the discovery of genes encoding the mitochondrial proteins Cpn60, PNT, mt-hsp70, ADP/ATP transporter, and Cpn10 (2, 7, 8, 21, 46) indicated that *E. histolytica* is the secondary “amito-
chondriate." While only a half-dozen proteins were shown to be localized to mitosomes (1, 2, 7, 8, 19, 21, 42, 44, 46), we recently discovered by proteomic analysis of isolated mitosomes that sulfate activation is the major pathway compartmentalized in mitosomes. Three enzymes consisting of the pathway and additional proteins required for the pathway, including sodium/sulfate symporter, mitochondrion carrier family protein, and chaperons, were identified in the mitosomal proteome. Although PNT was not discovered in the proteome, it was previously postulated to be mitochondrial-targeting peptide. However, it is important that the putative TS of EhPNT is certainly not a canonical mitochondrion-targeting peptide because it lacks basic residues such as arginine or lysine.

Despite the premise, we showed, in this study, that EhPNT was distributed to the membrane of vesicles and vacuoles, including lysosomes, phagosomes, and endosomes, but not to mitosomes. In addition, we showed that GFP fused with the amino-terminal putative TS of EhPNT was distributed to the cytosol, which disproved the premise that the amino-terminal domain of EhPNT functions as a putative organelle-targeting sequence. The 15_aa-long amino terminus of Cpn60 was not sufficient for the targeting of either luciferase or GFP to mitosomes (1; our unpublished data), although the removal of the first 15 aa of Cpn60 caused a mislocalization of the protein in the cytoplasm (42). Therefore, a role of the amino-terminal transit peptide for mitosomal transport remains obscure.

The association of PNT with the acidified compartments (lysosomes and phagosomes) (Fig. 5) is unprecedented in eu- karyotes, where PNT is usually localized to the inner mem- brane of mitochondria (48). The dependence of transhydrogenase activity of EhPNT on pH was demonstrated; the rate of transhydrogenation was higher at an acidic pH (5.5) than at a neutral pH (7.0 to 8.0) (49). The generation of NADPH by membrane-associated PNT (49) depends upon a proton-mo- toive force, which is likely generated by V-ATPase localized to the acidified compartment and also the nonacidified compart- ment. While the colocalization of V-ATPase and EhPNT has not been directly demonstrated, phagosomes contained major components of V-ATPase, as shown by the proteomic analysis of purified phagosomes (32, 33). Therefore, it is conceivable that amebic PNT localized in the acidic environment possesses enzymological properties suitable for acidic environments. Since our attempt to repress EhPNT expression by gene silencing failed (our unpublished data), the physiological role of EhPNT has not been elucidated. However, EhPNT may be involved in the detoxification of reactive oxygen and nitrogen species by supplying NADPH as a reducing power by using a proton gradient across the lysosomal and phagosomal mem- branes. During tissue invasion, E. histolytica adapts to changing oxygen tensions as it goes from the anaerobic colonic lumen to an oxygen-rich environment in the tissue (40). Additionally, the parasite must cope with cytocidal reactive oxygen and nitrogen species that are produced and released by activated phagocytes that are attracted to the site of infection (4, 20, 40).

Although we cannot exclude the possibility that truncated EhPNT was misfolded and aggregated in the cell, our immuno- fluorescence and cellular fractionation data are consistent with the premise that all truncated EhPNT was retained in the ER, which is suggestive of the default mechanisms of retention of multiple transmembrane proteins in the ER. The possibility that truncated EhPNT is retained in the heavy microsomal fraction was previously suggested (1), where the majority (93%) of luciferase activity of the recombinant protein consisted of the 67(aa-long amino-terminal portion of PNT fused to firefly luciferase was associated with the mixed membrane fraction and was as susceptible to trypsin degradation as cyto- solic luciferase from control parasites. Our present data also support the interpretation of the study that the fusion protein is embedded in the ER membrane and not targeted to mito- somes. We also showed that the aberrant mobility of PNT on SDS-PAGE gels (Fig. 1) was not due to either N-linked or O-linked glycosylation but was due to the hydrophobic na- ture of EhPNT. Altogether, E. histolytica PNT represents a novel class of PNT localized to lysosomes and appears to have evolved uniquely in this organism.

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