

## Localization and Targeting of an Unusual Pyridine Nucleotide Transhydrogenase in *Entamoeba histolytica*<sup>∇</sup>

Mohammad Abu Yousuf,<sup>1,2</sup> Fumika Mi-ichi,<sup>1</sup> Kumiko Nakada-Tsukui,<sup>1</sup> and Tomoyoshi Nozaki<sup>1\*</sup>

Department of Parasitology, National Institute of Infectious Diseases, Tokyo 162-8640, Japan,<sup>1</sup> and Department of Parasitology, Gunma University Graduate School of Medicine, 3-39-22 Showa-machi, Maebashi 371-8511, Japan<sup>2</sup>

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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 (*Eh*PNT) antibody raised against the NADH-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 NADPH formation from NADH (14, 15, 39) according to the reaction  $H^+_{out} + NADH + NADP^+ \leftrightarrow H^+_{in} + NAD^+ + NADPH$ , where “out” and “in” denote the cytosol and the matrix of the mitochondria, or the periplasmic space and the cytosol of bacteria, respectively.

PNT has been identified in several protozoan parasites, including *Entamoeba histolytica* (8, 51), *Eimeria tenella* (17, 47), *Mastigamoeba balamuthi* (11) *Plasmodium falciparum* (10), *Plasmodium yoelii* (6), and *Plasmodium berghei* (12). In general, PNT contains conserved structural units consisting of three domains, the NAD(H)-binding domain (domain I [dI]) and the NADP(H)-binding domain (domain III [dIII]), both of which face the matrix side of the eukaryotic mitochondria or the cytoplasmic side in bacteria, and the hydrophobic domain (domain II [dII]), containing 11 to 13 transmembrane regions. PNT from *E. tenella* and *E. histolytica* exists as a single polypeptide in an unusual configuration consisting of dIIb-dIII-dI-dIIa, with a 38-amino-acid-long linker region between dIII and dI (48).

*E. histolytica*, previously considered an “amitochondriate”

protist, is currently considered to possess a mitochondrion-related organelle with reduced and divergent functions, the mitosome (1, 21, 23a, 26, 42). Our recent proteomic study of isolated mitosomes identified about 20 new constituents (26), together with four proteins previously demonstrated in *E. histolytica* mitosomes: Cpn60 (8, 19, 21, 42), Cpn10 (46), mitochondrial Hsp70 (2, 44), and mitochondrion carrier family (MCF) (ADP/ATP transporter) (7). Despite the early presumption of PNT being localized in mitosomes (8), based on the amino-terminal region rich in hydroxylated (five serines and threonines) and acidic (three glutamates) amino acids, which slightly resembles known mitochondrion- and hydrogenosome-targeting sequences (8, 35), PNT was not discovered in the mitosome proteome. We also doubted this premise because PNT was one of the major proteins identified in isolated phagosomes (32, 33). Thus, the intracellular localization and trafficking of PNT remain unknown.

In this report, we showed that *E. histolytica* PNT (*Eh*PNT) is localized to various vesicles and vacuoles, including lysosomes and phagosomes, using wild-type amoebae and antiserum raised against recombinant *Eh*PNT and an *E. histolytica* line expressing *Eh*PNT with a carboxyl-terminal hemagglutinin (HA) epitope tag and anti-HA antibody. We also showed that all domains of *Eh*PNT are required for its trafficking to the acidic compartment by using amoeba transformants expressing the HA tag or green fluorescent protein (GFP) fused with a region containing various domains of *Eh*PNT.

### MATERIALS AND METHODS

**Cells, cultures, and reagents.** Trophozoites of *E. histolytica* strain HM-1:IMSS cl6 were maintained axenically in Diamond's BI-S-33 medium (9) at 35.5°C.

\* Corresponding author. Mailing address: Department of Parasitology, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku, Tokyo 162-8640, Japan. Phone: 81 3 5285 1111, ext. 2600. Fax: 81 3 5285 1219. E-mail: nozaki@nih.go.jp.

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Chinese hamster ovary (CHO) cells were maintained in F12 medium (Invitrogen, San Diego, CA) supplied with 10% fetal calf serum (Medical Biological Laboratory International, Woburn, MA) at 37°C with 5% CO<sub>2</sub>. *Escherichia coli* strains DH5 $\alpha$  and BL21(DE3) were purchased from Life Technologies (Tokyo, Japan) and Novagen (Madison, WI), respectively. LysoTracker Red DND-99 and CellTracker Orange CMTMR [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 dI (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'-CGAGGATCCGATGTATTATTGGTATCCAA AAG-3' and 5'-CGTTCTCGAGTCATCTTCTTCAGTTGAAAGA-3', respectively, where boldface type indicates the BamHI or XhoI site. The PCR products were cloned into the BamHI- 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-terminal region encompassing the putative targeting sequence (TS)+dIib (aa 1 to 330), TS+dIib+dIII (aa 1 to 525), TS+dIib+dIII+linker (aa 1 to 564), and TS+dIib+dIII+ linker+dI (aa 1 to 960) was amplified from an *E. histolytica* cDNA library by using a pair of appropriate primers and cloned into the BglIII site of pEhExHA (29). The antisense primers were 5'-CGTGGATCCATGAAA CATTTC AACATTCTG-3', 5'-CGTGGATCCGAATGATCTATTCATAGCT TTAC-3', 5'-CGTGGATCCCTTCTTCAATTAATCTTCAAATCCTTTC-3', 5'-CGTGGATCCATCTTCTGCAAGAAGAACTTTGGTGA-3', and 5'-CGTGG ATCCTTCTTCTTCAGTTGAAAGAGT-3', respectively, where boldface type represents the BamHI site. The sense primer described above was used for these full-length or truncated forms of *EhPNT* to express in the amoeba. To generate a plasmid to express GFP fused to the *EhPNT* TS, a pair of oligonucleotides corresponding to the TS were generated, self-annealed, and cloned into the BglIII site of pEhExGFP. The oligonucleotides were 5'-GATCTATGAGCACAAAGTT CTAGTATTGAAGAAGAAGTGTTC AATTATA-3' and 5'-GATCTATAATT GAACACTTCTTCTTCAACTAGAACTTGTGCTCATA-3', where boldface type represents the truncated BglIII site. pEhExGFP was generated by the ligation of the GFP protein-coding region of pKT-MG (29) into the BglIII-XhoI site of pEhEx (31). These constructs allowed the expression of PNT with the three tandem copies of the HA tag or GFP at the carboxyl terminus. The resulting plasmids were designated pPNTFL-HA, pTS/Iib-HA, pTS/Iib/III-HA, pTS/dIib/dIII/L-HA, pTS/Iib/III/L-HA, and pPNTS-GFP. The production of the Cpn60-HA transformant was previously described (26).

**Amoeba transformation.** Plasmids generated as described above were introduced into amoeba trophozoites by lipofection as previously described (30). Geneticin (Invitrogen, San Diego, CA) was added at a concentration of 1  $\mu$ g/ml at 24 h after transfection, and the Geneticin concentration was gradually increased for approximately 2 weeks until it reached 10  $\mu$ g/ml.

**Recombinant protein production.** pHisPNTdI was introduced into BL21(DE3) cells. The expression of the histidine-tagged *EhPNTdI* protein was induced with 1 mM isopropyl- $\beta$ -thiogalactoside at 37°C for 3 h. After harvesting and washing three times with phosphate-buffered saline (PBS) (pH 7.4), the bacteria were lysed in B-PER reagent (Pierce, Rockford, IL) containing Complete Mini EDTA-free protease inhibitor cocktail (Roche Diagnostic, Mannheim, Germany) and mixed with 1.0 ml of a 50% slurry of Ni<sup>2+</sup>-nitrilotriacetic acid (NTA) His-Bind resin. The recombinant *EhPNTdI*-bound resin was washed in a column three times with 25 ml of buffer A (50 mM NaH<sub>2</sub>PO<sub>4</sub>, 300 mM NaCl [pH 8.0]) containing 20 mM imidazole. Bound proteins were eluted with buffer A containing 1 M imidazole and dialyzed against PBS.

**Antibodies.** Anti-*EhPNT* antibody was raised against purified *EhPNTdI* in rabbit commercially (Operon, Tokyo, Japan). Anti-HA 11MO mouse monoclonal antibody was purchased from Berkeley Antibody (Berkeley, CA). Anti-*EhSec61*  $\alpha$ -subunit and anti-*E. histolytica* dolicol-P-mannose synthase (*EhD-PMS*) antibodies were a gift from Rosana Sánchez-López (37). Anti-galactose/*N*-acetylgalactosamine inhibitable lectin (Hgl) monoclonal antibody (3F4) (23) was a gift from Barbara J. Mann and William 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  $\times$  10<sup>6</sup> cells of *EhPNT*-HA- and Cpn60-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 mg/ml E-64). The soluble lysate, after centrifugation at 15,000  $\times$  g, was incubated with protein G-Sepharose beads (30  $\mu$ l of a 50% slurry) (Amersham Biosciences, Uppsala, Sweden) premixed with anti-HA antibody (0.5  $\mu$ l) 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) at 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  $\mu$ g/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)-anisole (3:2, vol/vol [100  $\mu$ l]) was added, and the samples were incubated for 4 h at 4°C under N<sub>2</sub> according to a method described previously (13). The reaction was stopped by slowly adding 200  $\mu$ l ice-cold H<sub>2</sub>O-pyridine (73:10, vol/vol) containing 0.1% SDS to the mixture. Anisole was extracted three times with 250  $\mu$ l ethyl ether. Dialysis 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 amidase that cleaves between the innermost *N*-acetylglucosamine and asparagine residues of asparagine-linked glycoproteins, according to the manufacturer's instructions.

**Immunofluorescence assay and organelle 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*  $\alpha$ -subunit (1:30), anti-*EhDPMS* 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  $\mu$ M CellTracker Orange or 20  $\mu$ M 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 isoforms 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\_014030, corresponding to GenBank accession numbers XP\_001914099 and AAC41577, respectively). They showed 90% mutual amino acid identity. The latter *EhPNT* isoform (EHI\_014030) 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)

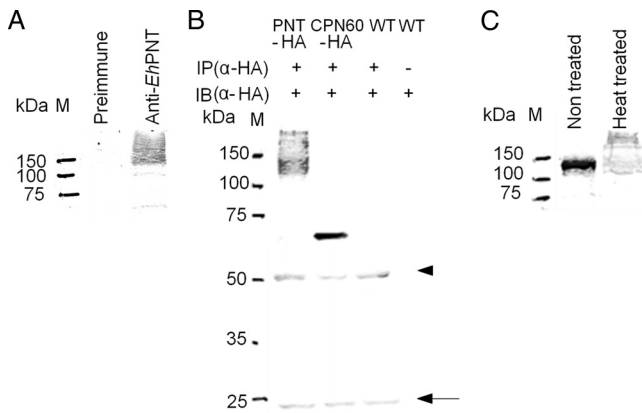


FIG. 1. Expression of PNT in *E. histolytica*. (A) Immunoblot analysis of native *EhPNT*. Approximately 10  $\mu$ g of total lysate was electrophoresed on a 12% SDS-polyacrylamide gel and subjected to an immunoblot assay with anti-*EhPNT* antibody or preimmune serum. M, molecular mass marker. (B) Immunoprecipitation 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* on an SDS-PAGE gel. Approximately 10  $\mu$ g 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.

(8). The dIII, dI, and dIIa regions of EHI\_055400 (1,098 aa) are conserved except for a single amino acid substitution in dIII. EHI\_055400 also contains a 5-aa extension at the amino terminus (MSLLL) and 9 aa substitutions in the putative TS (aa 1 to 14 of EHI\_014030) (data not shown). In addition, dIIb of EHI\_055400 contained 65 aa substitutions and two (4- and 6-aa-long) block insertions; the linker region also contains 24 aa substitutions. Since the domains involved in catalysis in EHI\_055400 and EHI\_014030 are totally conserved, and the two genes are expressed at comparable levels as steady-state mRNA by quantitative reverse transcriptase PCR (data not shown), we further studied only EHI\_014030 in the present work. EHI\_001930 (GenBank accession number XP\_653216), which is annotated as the PNT  $\beta$ -subunit, showed no significant homology to either of the two *EhPNT*s described above, while this sequence showed similarity with the  $\beta$ -subunit from other organisms and, thus, was excluded from this study.

**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. Immunoprecipi-

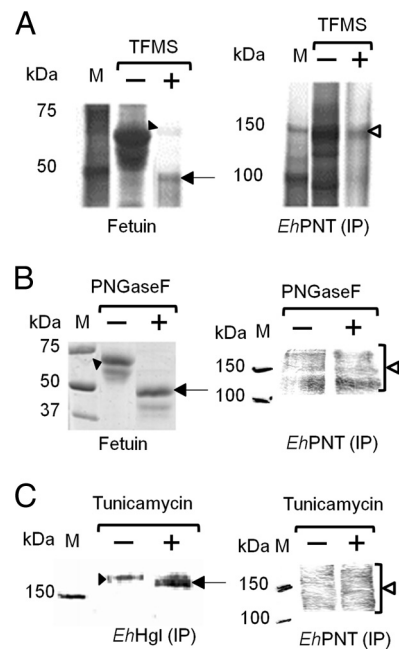


FIG. 2. Deglycosylation of *EhPNT*. (A and B) Deglycosylation by TFMS or PNGase F. The lysates obtained from the transformant expressing *EhPNT*-HA were subjected to immunoprecipitation (IP) with anti-HA antibody, followed by TFMS or PNGase F treatment and immunoblot detection with anti-HA antibody (right). 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*.

tated *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 $\times$  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-

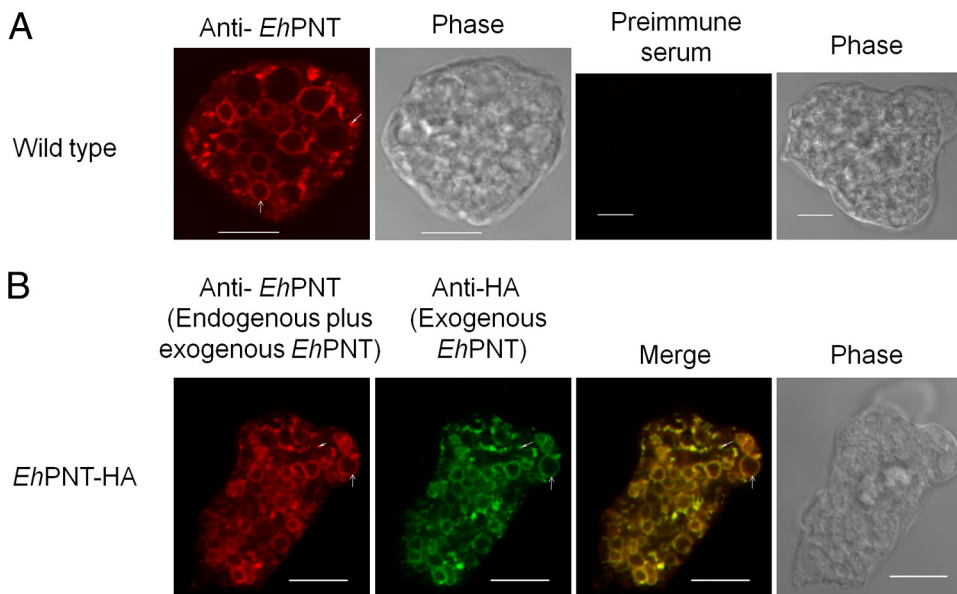


FIG. 3. (A) Subcellular localization of *EhpPNT* in wild-type amoebae. Wild-type amoebae were fixed, and an immunofluorescence assay was performed by using anti-*EhpPNT* (red) and preimmune sera. Bar, 10  $\mu$ m. Arrows and arrowheads indicate representative vacuolar and dot-like structures of *EhpPNT*, respectively. (B) Colocalization of endogenous and exogenous epitope-tagged *PNT* in *E. histolytica*. *EhpPNT*-HA-overexpressing amoebae were fixed, and an immunofluorescence assay was performed by using anti-*EhpPNT* (red) and anti-HA (green) antibodies.

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 *EhpPNT* on SDS-PAGE is likely due to the hydrophobic nature of the protein.

**Subcellular distribution of *EhpPNT*.** An immunofluorescence assay using anti-*EhpPNT* antibody showed that *EhpPNT* 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 *EhpPNT* using the amoebic transformant that expressed *EhpPNT* (EHI\_014030) with the carboxyl-terminal HA tag. The pattern of exogenous *EhpPNT*-HA (EHI\_014030) and that of endogenous *EhpPNT* (a sum of EHI\_055400 and EHI\_014030) plus exogenous *EhpPNT* (EHI\_014030) were indistinguishable (Fig. 3B). Since *EhpPNT* was previously postulated to be localized to mitochondria (8), we next examined the localization of *EhpPNT* and *EhCpn60*, the authentic marker of mitochondria, in the amoebic transformant expressing *EhCpn60*-HA (26) using

anti-*EhpPNT* and anti-HA antibodies. No colocalization of *EhpPNT* and *EhCpn60* was observed (Fig. 4).

Since *EhpPNT* was previously detected in isolated phagosomes (32, 33), we examined the localization of *EhpPNT* 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-*EhpPNT* antibody (Fig. 5A) showed that the phagocytosed CHO cells were associated with *EhpPNT* 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 ( $65\% \pm 6\%$ ,  $79\% \pm 9\%$ , and  $80\% \pm 8\%$  at 10, 30, and 60 min, respectively). We then examined whether *EhpPNT* 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 *EhpPNT* under steady-state conditions ( $79\% \pm 6\%$  association) (Fig. 5B). To see whether *EhpPNT* is also associated with endosomes, we examined the localization of an endocytosed

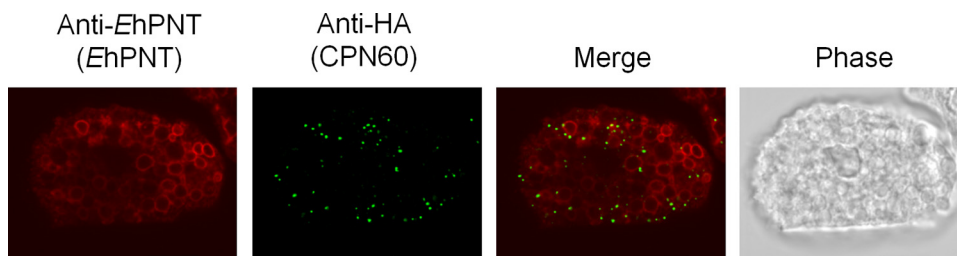


FIG. 4. Lack of association of *EhpPNT* with mitochondria. The amoebic transformant expressing *EhCpn60*-HA was stained with anti-HA (green) and anti-*EhpPNT* (red) antibodies to visualize *EhCpn60* and *EhpPNT*, respectively. Bar, 10  $\mu$ m.

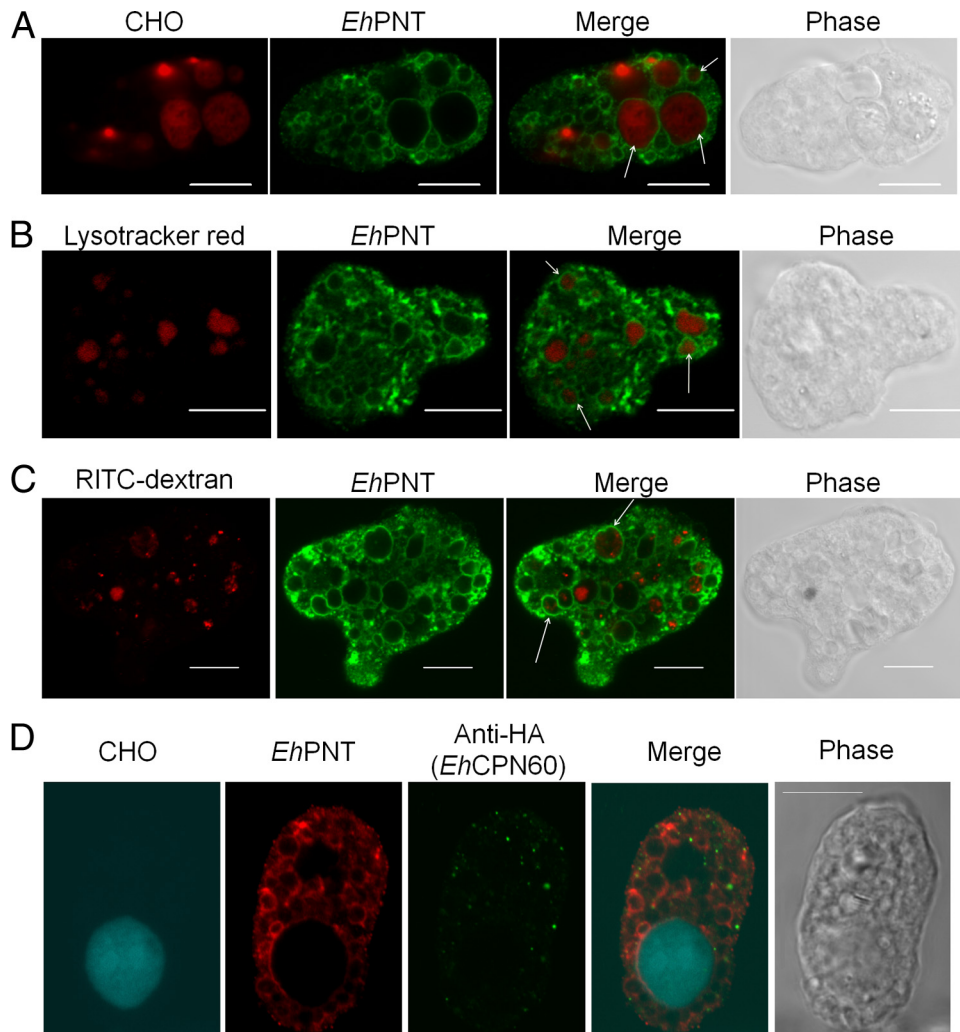


FIG. 5. Localization of *EhPNT* to phagosomes, lysosomes, and endosomes. (A) Association of *EhPNT* with phagosomes. Amoebae were incubated with CellTracker Orange-loaded CHO cells (red) for 60 min, fixed, and reacted with anti-*EhPNT* antibody (green). Arrows indicate representative phagocytosed CHO cells associated with *EhPNT*. (B) Association of *EhPNT* with lysosomes. Amoebae were labeled with LysoTracker (red) and subjected to an immunofluorescence assay with anti-*EhPNT* antibody (green). Arrows indicate representative lysosomes associated with *EhPNT*. (C) Association of *EhPNT* with the fluid-phase marker. Amoebae were incubated with medium containing RITC-dextran (red) for 1 h. The cells were fixed and reacted with anti-*EhPNT* antibody (green). Arrows indicate representative endocytosed RITC-dextran associated with *EhPNT*. (D) Subcellular localization of phagosomes, mitosomes, and *EhPNT*. The amoebic transformant expressing *EhCpn60*-HA was incubated with CellTracker Blue-loaded CHO cells (blue) for 60 min, fixed, and reacted with anti-*EhPNT* (red) and anti-*EhCpn60* (green) antibodies. Bar, 10  $\mu$ m.

fluid-phase 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 pro-

tein 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 MSTSSSIEEEVFNY appeared to contain the elements implicated for the TS (rich in hydroxylated and hydrophobic amino acids). The transformants expressing TS+dIIb-HA, TS+dIIb+dIII-HA, TS+dIIb+dIII+L-HA, or TS+dIIb+dIII+L+dI-HA showed a distribution that overlapped that of the endoplasmic reticulum (ER), visualized with anti-*EhSec61*  $\alpha$ -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*  $\alpha$ -subunit or anti-*EhDPMS* antibody. Fractionation of the amoeba lysate followed by immunoblot analysis with anti-HA or anti-GFP an-

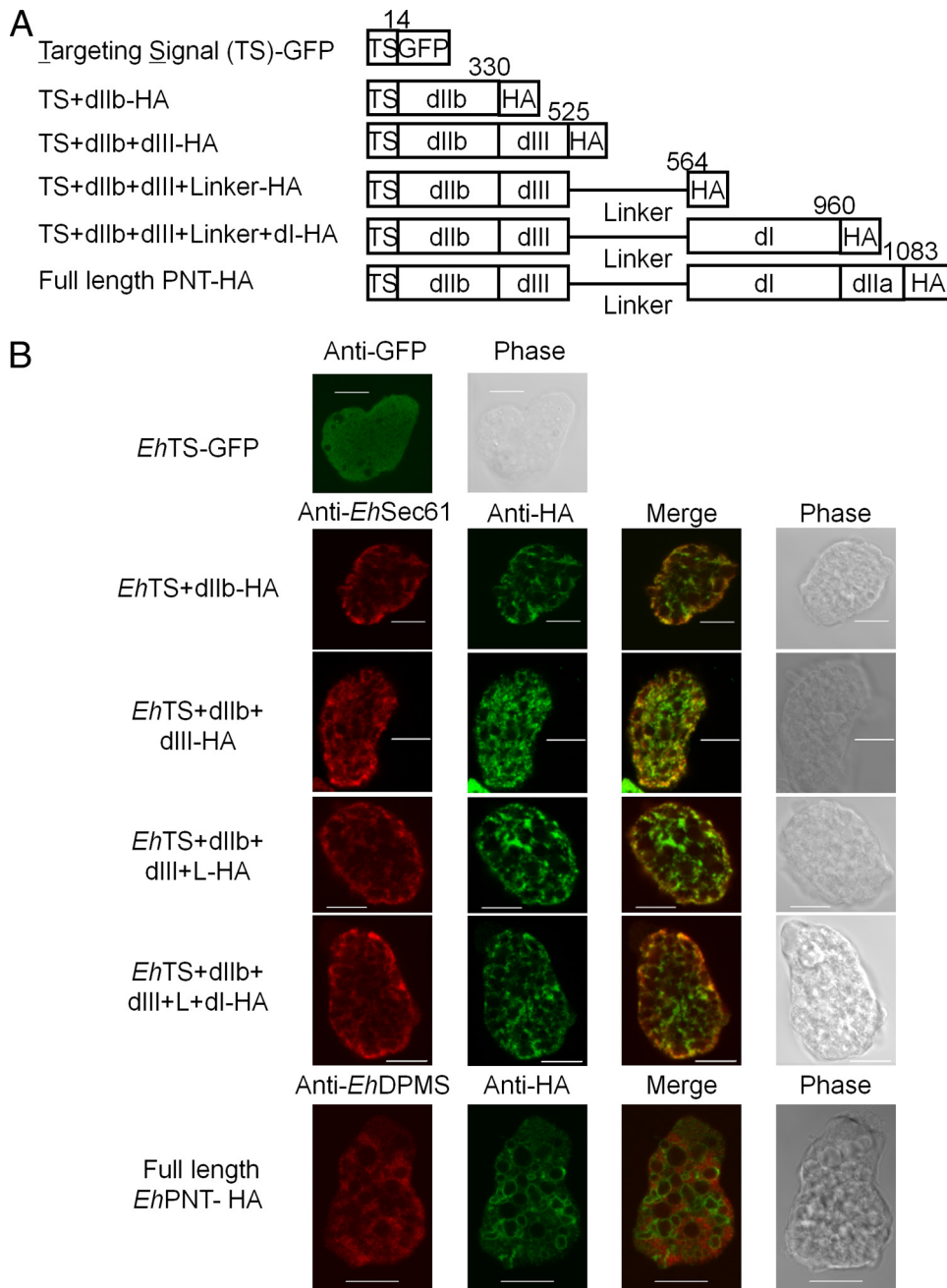


FIG. 6. Localization of a series of truncated *EhPNT* proteins. (A) Schematic representation of HA epitope-tagged or GFP-fused recombinant *EhPNT* used in the study. Domains, epitope (or GFP), and amino acid numbers are shown. (B) Localization of epitope-tagged or GFP-fused carboxyl-terminally truncated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

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

**DISCUSSION**

Mitosomes have been identified in several parasitic protozoan lineages such as *E. histolytica*, *Giardia intestinalis*, *Trachipleisto-*

*phora 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 mitosomal based on the resemblance of the amino-terminal region of *EhPNT* to the potential mitochondrion-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 eukaryotes, where PNT is usually localized to the inner membrane 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-motive force, which is likely generated by V-ATPase localized to the acidified compartment and also the nonacidified compartment. 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 membranes. 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 cytotoxic 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 immunofluorescence 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 cytosolic 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 mitosomes. We also showed that the aberrant mobility of PNT on SDS-PAGE gels (Fig. 1) was not likely due to either N-linked or O-linked glycosylation but was due to the hydrophobic nature 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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