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Molecular cloning and characterization of ouabain-insensitive Na^+-ATPase in the parasitic protist, *Trypanosoma cruzi*

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Abstract

Maintaining low intracellular sodium concentrations is vital for almost all organisms. Na^+ efflux is generally governed by P-type ATPases, Na^+/K^+-ATPase in animals and Na^+-ATPase, called ENA, in fungi and plants. *Trypanosoma cruzi*, which parasitizes mammalian cells, must undergo drastic adaptations to high Na^+ concentrations outside and low Na^+ concentrations inside host cells. However, *T. cruzi* Na^+ efflux pumps have not been identified. We report here the cloning and characterization of the gene encoding Na^+-ATPase in *T cruzi*, which resembled fungal and plant *ENA*s, termed *TcENA*. TcENA was a plasma membrane protein expressed throughout the parasite life cycle. The transcription level of *TcENA* was higher in insect stage epimastigotes and blood stream trypomastigotes than in intracellular amastigotes, probably reflecting the high Na^+ concentration outside the host cells. Biochemical analysis of TcENA expressed heterologously in mammalian cells demonstrated, for the fist time, that the ATPase activity of TcENA is stimulated by both Na^+ and K^+ and is insensitive to ouabain, a specific inhibitor of Na^+/K^+-ATPases. Furthermore, epimastigotes overproducing TcENA showed increased tolerance to high Na^+ stress. Our findings suggest that TcENA acts as a sodium pump and provide insights into the regulation of ion homeostasis in the parasitic protist.

Keywords: *Trypanosoma cruzi*; P-type ATPase; ENA; Ouabain; Heterologous expression; Sodium tolerance

1. Introduction

Trypanosoma cruzi is the etiological agent of Chagas' disease, which affects about 17 million people in Latin America [1]. Moreover, about 25% of the population has been estimated to be at risk [1]. *T. cruzi* has three main developmental stages: the epimastigote, which multiplies in the intestinal cavity of triatomine insects; the amastigote, which propagates by binary fission in the cytosol of mammalian cells; and the trypomastigote, the infective stage of the parasite that circulates in the blood stream of mammalian hosts [2].

Remarkably, the amastigote and trypomastigote must survive in extremely different ionic environments. That is, the trypomastigote lives in an environment containing high Na^+ (135–146 mM) and low K^+ (3.5–5.5 mM) concentrations, whereas the amastigote lives in an environment containing low Na^+ (5–15 mM) and high K^+ (140–155 mM) concentrations [3]. Thus, regulation of Na^+ and K^+ concentrations is important for parasite adaptation to the host environments and for parasite survival.

P-type ATPases are membrane proteins that can transport specific ions, including Na^+, K^+, Ca^{2+}, and H^+, across membranes against concentration gradients. Formation of a phosphorylated intermediate is characteristic of this type of enzyme during its reaction cycle [4]. P-type ATPases are responsible for maintaining various ion milieus, including the maintenance of low (<30 mM) intracellular sodium concentrations. This property is essential for all living organisms, because high internal sodium is generally toxic [5]. In mammalian cells, an ouabain-sensitive Na^+/K^+-ATPase, which exports three Na^+ ions coupled with the import of two K^+ ions per cycle, establishes an electrochemical gradient for Na^+, which drives

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the cotransport of various solutes, including glucose, Pi, and amino acids, by symporters [6].

In plants and fungi, Na^+ efflux is essentially driven by a different type of Na^+-ATPases, called ENA [7,8]. *Saccharomyces cerevisiae* ENA has been shown to mediate tolerance to high Na^+ concentrations, and disruption of the *ENA* gene resulted in loss of viability under this condition [9]. Recently, the moss, *Physcomitrella patens*, was reported to possess two isoforms of an ENA-type ATPase (PpENA), and complementation of the *ENA*-disrupted yeast mutants with PpENA was found to rescue the defective phenotype of the former [10].

On the other hand, in parasitic protists, including *T. cruzi*, the molecular mechanism for the maintenance of intracellular Na^+ and K^+ concentrations remains unclear. In *T. cruzi*, the presence of ouabain-insensitive, Na^+-stimulated ATPase activity has been suggested but not been molecularly identified [11]. Here, we report the cloning of a gene encoding a P-type ATPase from *T. cruzi*, which is similar to ENA and termed TcENA. Biochemical analyses using a mammalian cell expression system revealed that TcENA is an ouabain-insensitive ATPase and that its activity is stimulated by both Na^+ and K^+. In addition, *T. cruzi* epimastigotes overexpressing TcENA showed significant tolerance to high Na^+ concentrations in the culture medium. Our findings provide insights into the mechanism by which the various developmental stages of *T. cruzi* maintain homeostasis to monovalent cations.

2. Materials and methods

2.1. Parasite materials

HT1080 cells, a human fibrosarcoma cell line, were infected by the mammalian stages of *T. cruzi* Tulahuen strain as described [12]. HT1080 cells, with or without *T. cruzi*, were inoculated at an initial density of $3–5\times10^5$/ml into DMEM (Sigma-Aldrich Japan, K.K., Japan) supplemented with 10% fetal bovine serum (FBS) in 25-cm^2 culture flasks and subcultured every 3–4 days. Trypomastigotes were collected from the subculture of infected HT1080 cells by centrifugation at 800×*g* for 5 min at 4 °C in 15-ml polypropylene tubes to remove host cells and cell debris. The resulting supernatant was centrifuged at 1500×*g* for 10 min at 4 °C, and the pellet containing trypomastigotes was washed three times with 10 ml DMEM by repeated suspension and centrifugation. The purified trypomastigotes were counted on an improved Neubauer hemocytometer and each preparation was verified to contain less than 10% amastigotes. Epimastigotes were routinely subcultured weekly in LIT medium (No. 1029, ATCC medium formulations) supplemented with 10% FBS and 10 μg/ml of hemin (Sigma-Aldrich Japan) by seeding epimastigotes at an initial density of 5×10^5/ml in tightly capped 25-cm^2 culture flasks at 26 °C.

2.2. Cloning of T. cruzi P-type ATPase gene

To obtain a gene encoding *T. cruzi* P-type ATPase, we designed degenerate primers (forward; 5′-ATGAARGGNGCNCCNGA-3′, reverse; 5′-GGRTGRTC-NCCNGTNACCAT-3′) to amplify the conserved regions of P-type ATPases, which target for the 507–621 amino acids of the human Na^+/K^+ ATPase. PCR was performed using these primers, oligo-dT-primed cDNA from trypomastigotes, and ExTaq DNA polymerase (Takara Bio Inc., Otsu, Shiga, Japan). The amplification protocol consisted of 30 cycles of denaturation at 94 °C for 30 s, annealing at 50 °C for 30 s, and extension at 72 °C for 1 min. The 370-bp PCR product was subcloned into a pCR II-TOPO vector (Invitrogen, Carlsbad, CA, USA) and sequenced, and shown to encode a portion of P-type ATPase, similar to *Saccharomyces cerevisiae ENA1*. The PCR product was labeled with digoxigenin (DIG) using DIG-PCR labeling kit (Roche Diagnostics K.K., Minato-ku, Tokyo, Japan) and the primers (forward; 5′-CGTGTAATTGAT-CTTTGCAC-3′, reverse; 5′- ACCACAATACCGGCATGTTG-3′). The DIG-labeled DNA probe was used to screen a *T. cruzi* genomic library [13], and a single positive clone containing the 18-kb insert DNA was obtained and shown to contain an open reading frame for the *T. cruzi* P-type ATPase gene. A hydropathy plot of the predicted amino acid sequence of TcENA was obtained using the TMpred program (http://www.ch.embnet.org/software/TMPRED_form.html).

2.3. Preparation of polyclonal antisera specific to TcENA

Using the primers, 5′-CGGGATCCATGTTGGAACTGCCGCCAG-3′ (sense) and 5′-CGGGATCCTCACGCCAAAACGCTCCAC-3′ (antisense), and KOD-Plus-DNA polymerase (High-Fidelity type, Toyobo Co., Ltd., Osaka, Japan), the central cytoplasmic region of *TcENA* was amplified by PCR. The PCR product was digested with *Bam*HI, subcloned into the corresponding site of the expression vector pET28a (Takara Bio), and confirmed to be free of PCR-generated errors by sequencing. The recombinant plasmid was used to transform BL21-AI™ competent *E. coli* (Invitrogen), and expression of recombinant TcENA was carried out under the conditions recommended by the manufacturer. Expression was induced by incubating the cells in 1 mM isopropyl-β-D-thiogalactopyranoside. The bacterial cells were lysed by sonication and precipitated by centrifugation at 10,000×*g* for 10 min at 4 °C. The resulting precipitate was solubilized in 8 M urea, incubated with TALON Metal Affinity Resin (BD Biosciences, San Jose, CA, USA), washed and eluted, as recommended. The resulting eluates were dialyzed extensively against phosphate-buffered saline (PBS), pH 7.2, re-precipitated, washed again with PBS, solubilized in 8 M urea/PBS, and stored at −80 °C. The purity of the recombinant TcENA was confirmed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) to be over 95%. For production of antibody specific to TcENA, BALB/c mice were each immunized with 50 μg recombinant TcENA in Freund's complete adjuvant and boosted once with 50 μg recombinant TcENA in Freund's incomplete adjuvant. Two weeks later, the immune sera were collected and used for Western blotting and indirect immunofluorescence analysis at appropriate dilutions.

2.4. Indirect immunofluorescence

T. cruzi infected HT1080 cells were grown on a cover slip in a well of a 12-well plate as described [14]. The parasite and host cells were fixed for 30 min in PBS containing 4% paraformaldehyde and permeabilized in 0.1% Triton-X 100/0.1% sodium citrate for 5 min at 4 °C. Blocking was performed in blocking solution (PBS containing 0.05% Tween 20 and 1% BSA) for 20 min, and the cells were incubated with TcENA-specific antisera in blocking solution for 18 h at room temperature. After washing with PBS, the cells were reacted with the anti-mouse FITC-conjugate, and TcENA localization was assessed under a fluorescent microscope.

2.5. Western blotting

Epimastigotes were washed with PBS and homogenized in the presence of protease inhibitor cocktail (Complete Mini, Roche Diagnostics) using cell disruption bomb (Parr Instrument Company, Moline, IL, USA) at 1,200 psi. After removal of the nuclei and cell debris by centrifugation at 1600×*g* for 10 min, the lysate was centrifuged at 150,000×*g* for 1 h. The resulting pellet and supernatant were defined as the membrane and cytosolic fractions, respectively. The proteins were separated by SDS-PAGE and blotted to PVDF membranes, which were incubated with TcENA-specific antibody. Bound primary antibodies were visualized with alkaline phosphatase-conjugated secondary antibody and CSPD (Roche Diagnostics).

2.6. Northern blotting

Total RNA was extracted from epimastigotes, trypomastigotes, or amastigotes using Trizol® reagent (Invitrogen) according to the manufacturer's recommendations, and Northern hybridization was performed as

described [12]. Briefly, the RNA samples were electrophoresed on 1% agarose gels, blotted to nylon membranes (Roche Diagnostics), and hybridized with the TcENA-specific DNA probe, which was DIG-labeled using PCR probe Synthesis Kit (Roche Diagnostics) in the presence of the primers, 5′-CACCATGTCGGATTCGAAAGAGC-3′ and 5′-TTAGCGGTA-CACCATATTCTTGC-3′, and the TcENA-carrying plasmid DNA as a template. Chemiluminescent detection of the hybridization signals was performed using CSPD (Roche Diagnostics).

2.7. Expression of TcENA

The full-length TcENA cDNA was PCR amplified using the primers, 5′-CTCGAGATGTCGGATTCGAAAGAGCT-3′ (sense) and 5′-TTTACC-GGTCCACGCCTCTTCTTTTTCCTCT-3′ (antisense), KOD-Plus-DNA polymerase, and cDNA synthesized from epimastigote total RNA. The PCR product was cloned into pCRII-TOPO and restricted with *Xho*I and *Age*I. The resulting cDNA fragment was cloned into the *Xho*I and *Age*I sites of the mammalian expression vector pEGFP-N1 (Clontech, Takara Bio Inc., Otsu, Shiga, Japan), which allows carboxyl terminal fusion with EGFP (pEGFP-TcENA). FreeStyle™ 293-F cells (Invitrogen) were cultured in 125-ml polycarbonate Erlenmeyer flasks containing 30 ml of FreeStyle™ 293 Expression medium to a density of 5×10^5 cells/ml. The cells were transfected with 30 μg pEGFP-TcENA using 40 μl of LipofectAMINE™ 2000 (Invitrogen) according to the manufacturer's instructions. The cells were incubated for 48 h and transferred to fresh medium supplemented with 1 mg/ml G418 sulfate (cell culture tested, Calbiochem, EMD Biosciences, Inc., La Jolla, CA, USA) to select transformed cells, changing the medium every 3 days. Four weeks after the start of continuous cultures, the transformed cells expressing EGFP were established and used for further experiments.

2.8. Measurement of ATPase activity

The FreeStyle™ 293-F cells expressing the TcENA-EGFP fusion protein were harvested by centrifugation at 100×*g* for 5 min, and suspended in 10 volumes of ice-cold homogenization buffer (250 mM sucrose, 50 mM imidazole–HCl (pH 7.0), 5 mM sodium azide, and 2 mM EDTA), and homogenized in a Teflon-coated Dounce homogenizer with 10 gentle strokes. The homogenate was centrifuged at 5000×*g* for 10 min to remove nuclei, mitochondria, and cell debris. The supernatant was centrifuged at 47,000×*g* for 30 min and the resulting supernatants and precipitates were designated the cytosolic and membrane fractions, respectively. The pellet was suspended in homogenization buffer containing 0.065% sodium deoxycholate (Sigma, St. Louis, MO, USA). ATPase activity was measured as described [15]. The reaction was initiated by adding 30 μg proteins to 1 ml assay mixture containing 50 mM Tris–HCl pH 7.6, 4 mM $MgCl_2$, 2 mM EGTA, 3 mM Tris–ATP, 1 mM phosphoenolpyruvate, 10 U/ml pyruvate kinase (Wako Pure Chemical Industries Co., Tokyo, Japan), 30 U/ml lactate dehydrogenase (Wako Pure Chemical Industries), 0.2 mM NADH, 10 nM bafilomycin (an inhibitor of V-type ATPase, Sigma), 100 nM tapsigargin (an inhibitor of sarcoplasmic reticulum Ca^{2+}-ATPase, Sigma), 5 mM sodium azide, 130 mM NaCl, and 20 mM KCl. Sodium orthovanadate (Calbiochem) and ouabain (Sigma) were used as inhibitors of P-type ATPase and Na^+/K^+-ATPase, respectively. Choline chloride (Sigma) was used to keep the ion strength in the assay mixture. The rate of reaction was determined as the steady-state value of dA_{340}/dt at 37 °C for 10 min. ATPase activity was calculated based on the extinction coefficient of NADH ($\varepsilon=6.22\ mM^{-1}\ cm^{-1}$).

2.9. Expression of EGFP-tagged TcENA in T. cruzi

The vector backbone of pEGFP-TcENA was replaced by that of the trypanosomatid shuttle vector, pTREX [16]. Briefly, The DNA fragment carrying the *TcENA-EGFP* fused gene was amplified from pEGFP-TcENA by PCR using the primers, 5′-AAACTAGTATGTCGGATTCGAAAGAGCT-3′ (sense) and 5′-TTCTCGAGTTAAGCGTAATCTGGAACATCGTATGGG-TACTTGTACAGCTCG-3′ (antisense), and KOD-Plus- DNA polymerase. The PCR product was subcloned into pCR II-TOPO, digested with *Xho*I, and cloned into the *Xho*I site of pTREX. The resulting recombinant plasmid, designated pTREX/TcENA-EGFP, was used for transformation of *T. cruzi* epimastigotes by electroporation using a Gene Pulser apparatus (Bio-Rad Laboratories, Hercules, CA, USA) with three pulses at 400 V and 500 μF [17]. The stable transformants were selected by incubating the cells for 60 days in LIT medium containing 500 μg/ml G418. Expression of TcENA-EGFP in the transformed epimastigotes was detected by fluorescent microscopy.

3. Results

3.1. Isolation and nucleotide sequence analysis of the TcENA gene

We designed degenerate primers based on the conserved amino acid sequences of animal Na^+/K^+-ATPases and fungal *ENA*-type ATPases. Using PCR, we obtained a product of approximately 370 bp, with a deduced amino acid sequence similar to those of fungal ENA and the calcium motive P-type ATPases of trypanosomatids (data not shown). This DNA fragment was DIG-labeled and used to screen a *T. cruzi* genomic library, resulting in the isolation of a clone containing an 18-kb insert encoding full-length *T. cruzi* P-type ATPase DNA, which we designated *TcENA*.

The open reading frame of *TcENA* consisted of 3,117 nucleotides, encoding 1,039 amino acids with a calculated molecular mass of 120 kDa (GenBank™ AB107891, Fig. 1). TcENA contains the sequence DKTGTLT, a motif of the phosphorylation site conserved in all P-type ATPases. The hydropathy plot of TcENA shows 10 hydrophobic regions of sufficient length to span the membrane, indicating the presence of ten transmembrane segments (Fig. 2).

The deduced amino acid sequence of TcENA shared identity with the sequences of the putative calcium motive P-type ATPases of *Leishmania donovani* (66% identity, GenBank™ AF067495) and *T. brucei* (71%, Tb09.244.2570 in; http://www.sanger.ac.uk/Projects/T_brucei/) and was identical to that of putative calcium motive P-type ATPase (Tc00.1047053510769.120 in; http://tcruzidb.org/). We identified an additional ortholog of TcENA (Tc00.1047053506241.70 in; http://tcruzidb.org/) in the *T. cruzi* genome database, which had a 99.3% amino acid sequence identity to that of TcENA.

The TcENA sequence was similar to those of various sodium-pumping ATPases, ENAs, including PpENA1 of the moss *Physcomitrella patens* (38% identity, GenBank™ AJ564254, see Fig. 1), cta3 of *Schizosaccharomyces pombe* (35%, GenBank™ J05634), and ENA1 of *Saccharomyces cerevisiae* (34%, GenBank™ U24069). Based on sequence homology, P-type ATPases can be divided into five groups [8]. Although the trypanosomatid ATPases were annotated as putative "calcium motive P-type ATPases" without biochemical analysis and their amino acid sequences are less identical to that of fungal and plant ATPases, the phylogenetic analyses suggested that the trypanosomatid, plant, and fungal ATPases are phylogenetically related and belong to type IID ATPases [10,18]. Thus, TcENA appears to be a type IID ATPase and is likely to play a role in sodium transport.

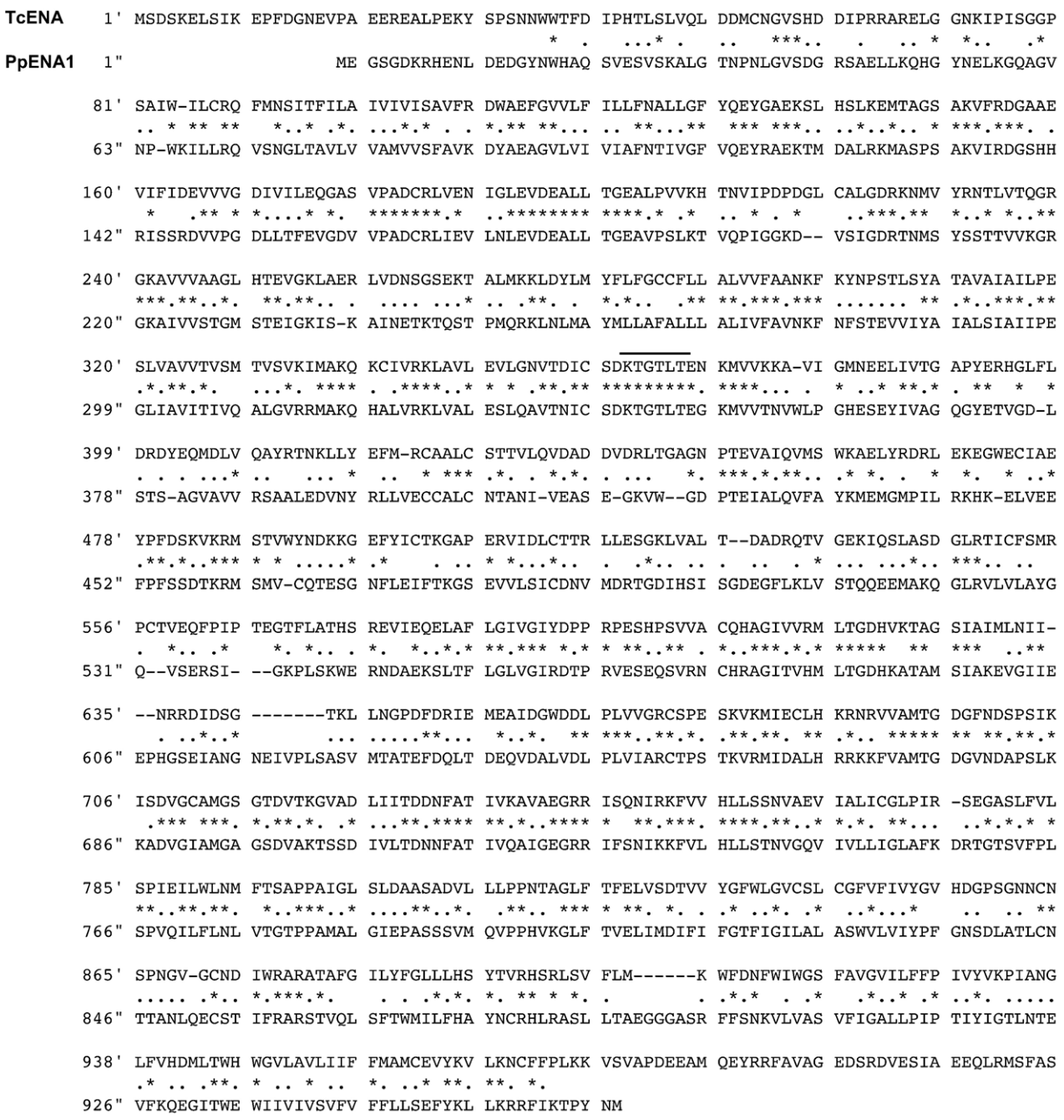

Fig. 1. Alignment of the deduced amino acid sequences of the ENA-type ATPases of *Trypanosoma cruzi* (TcENA, GenBank™ AB107891) and *Physcomitrella patent* (PpENA1, GenBank™ AJ564254). Asterisks and dots represent identical and equivalent amino acids, respectively. The phosphorylation site, characteristic of P-type ATPases, is underlined.

3.2. Transcription of TcENA gene at different stages of T. cruzi

To analyze transcription level of the *TcENA* gene, we performed Northern hybridization on total RNAs from the three *T. cruzi* stages, trypomastigotes, epimastigotes, and amastigotes, using the TcENA-specific DNA probe (Fig. 3). Since it is difficult to separate amastigotes from host cells, we used total RNA from heavily parasitized host cells as amastigote RNA (lane A, Fig. 3A).

The TcENA transcript was present as a band of approximately 4.5 kb in all three *T. cruzi* stages, with additional faint bands at higher molecular weight in epimastigote. There were no bands in host RNA (Fig. 3B). The transcription level of TcENA differed by developmental stage. When the amount of RNA was normalized relative to the signal intensity of *T. cruzi* 5S rRNA, the transcription level of TcENA was 1.8-fold higher in trypomastigotes than in amastigotes (Fig. 3B).

3.3. Localization of TcENA in the plasma membrane of T. cruzi

We investigated expression of TcENA in epimastigote by Western blotting. Since fungal ENA is localized on the plasma

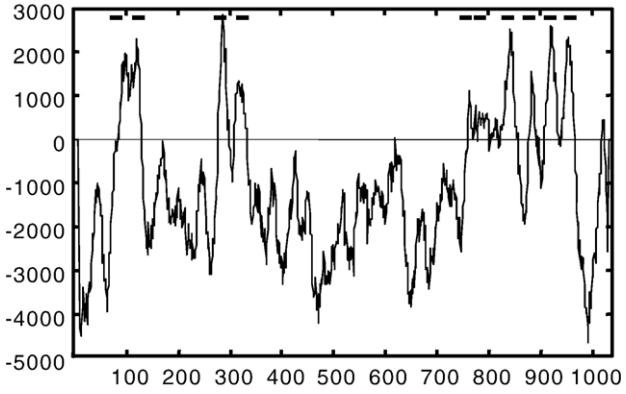

Fig. 2. Hydropathy plot of TcENA based on the TMpred program (http://www.ch.embnet.org/software/TMPRED_form.html). Possible transmembrane regions are indicated by the horizontal bars at the top of the frame.

membrane, we prepared cytosolic and membrane fractions of *T. cruzi* epimastigotes and reacted each with antisera raised against recombinant TcENA (Fig. 4A). We observed the expected 120 kDa band only in the membrane fraction, suggesting that, like other P-type ATPases, TcENA is expressed as a membrane protein.

We also assayed the subcellular localization of TcENA by indirect immunofluorescence in trypomastigotes (Fig. 4B) and amastigotes (Fig. 4C). In both developmental stages, the anti-TcENA antibody reacted predominantly with the parasite plasma membrane. In *S. cerevisiae*, ENA1 is required for the efflux of sodium ions and is localized in the plasma membrane [19]. These results suggest that TcENA is localized in the plasma membrane of *T. cruzi* and is most likely to function as an efflux pump of monovalent cations, thus maintaining ion homeostasis in the parasite.

3.4. Heterologous expression of TcENA in eukaryotic cells

As *S. cerevisiae* B31 strain (*ena1*Δ::*HIS3*::*ena4 nha1*Δ::*LEU2*) does not carry the two major Na^+ efflux systems, ENA

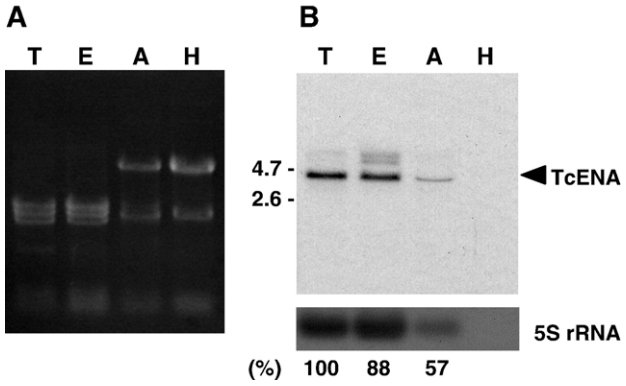

Fig. 3. Expression of *TcENA* mRNA. (A) Ten μg total RNA isolated from trypomastigotes (T), epimastigotes (E), amastigotes-infected cells (A), and host cells (H) were electrophoresed on agarose gels and stained with ethidium bromide. (B) RNAs of panel A were blotted to nylon membranes and hybridized with DIG-labeled DNA probes specific for the *TcENA* (upper panel) and *5S rRNA* (lower panel) genes. The molecular size in kilobases is indicated on the left. At the bottom, shown are the transcription levels of *TcENA*, normalized to *5S rRNA*.

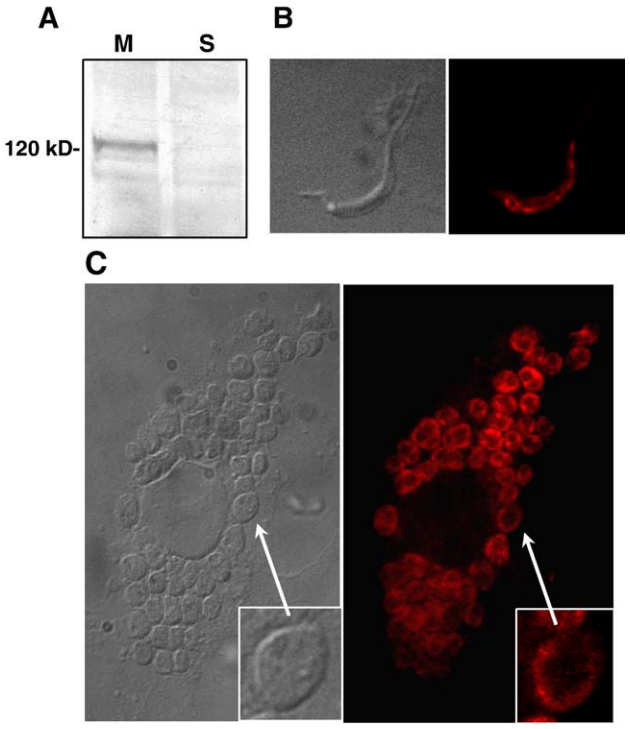

Fig. 4. Localization of TcENA in *T. cruzi*. (A) Membrane (M) and soluble (S) fractions were prepared from epimastigotes, resolved on SDS-PAGE, and reacted with anti-TcENA antibody. Six μg of the proteins were loaded onto each lane. kDa; kilodaltons. (B, C) Detection of TcENA in trypomastigotes (B) and intracellular amastigotes (C). The parasites were fixed, incubated with TcENA-specific antisera, and stained with FITC-labeled secondary antibodies (right panels). The left panels represent differential interference contrast (DIC) images. The inset in (C) indicates a single amastigote (an arrow).

and Na^+/H^+ antiporter, it is more sensitive to salt stress [20]. Expression of *P. patens* ENA in the B31 strain allowed survival of the transformants under conditions of high Na^+ [10]. Thus, we assayed whether expression of TcENA could complement the B31 phenotype. When we tried to express TcENA using pDR195, a plasmid that carries the *PMA1* promoter [21], as well as in various commercially available vectors, none of these transformants showed tolerance to high Na^+ (data not shown). In addition, when we attempted to transform *S. cerevisiae* G19 strain (*ena1*Δ::*HIS3*::*ena4*) [22] with *TcENA* gene, we failed to detect the phenotype (data not shown).

We therefore attempted to express TcENA using a mammalian expression system, in which FreeStyle™ 293-F cells, a derivative of the human 293 cell line, were transformed using the recombinant plasmid carrying the *TcENA-EGFP* fusion gene (Fig. 5). FreeStyle™ 293-F cells were transfected with the plasmid by lipofection and continuously cultured for 4 weeks in the presence of 1 mg/ml G418 to obtain stable transformants. In human cells, fluorescence of TcENA-EGFP was detected in the plasma membrane, indicating that TcENA-EGFP was successfully expressed as a plasma membrane protein (Fig. 5A). We therefore used this heterologous expression system to determine the enzymatic properties of TcENA.

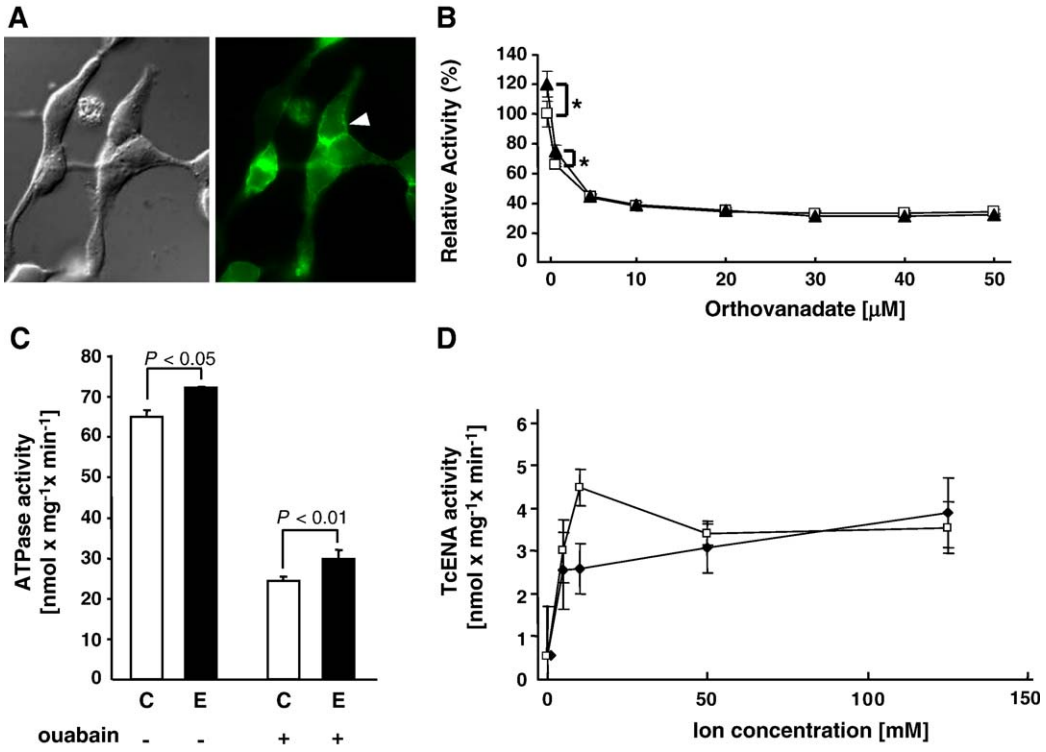

Fig. 5. Expression of TcENA in a human cell line. (A) Direct fluorescence localization of EGFP-tagged TcENA in the plasma membrane of FreeStyle™ 293-F cells. The arrowhead indicates a monolayer cell demonstrating fluorescence on its surface. Left, DIC image; right, fluorescence image. (B) Measurement of ATPase activity in the plasma membrane fraction of TcENA-expressing (triangle) and control (square) cells in the presence of sodium orthovanadate. Values are mean±S.D. of three independent experiments. An asterisk represents the statistical significance of $P<0.05$. (C) Measurement of ATPase activity in the plasma membrane fraction of TcENA-expressing (E) and control (C) cells in the absence or presence of 1 mM ouabain. (D) Effect of different concentrations of Na^+ (diamond) and K^+ (square) on TcENA activity in the presence of 1 mM ouabain. The TcENA-specific ATPase activity was shown by subtracting the activity of the control cells from that of the TcENA-expressing cells. The ionic strength was kept constant by adding choline chloride and the concentrations of choline plus either Na^+ or K^+ were adjusted to be 140 mM. Values are mean±S.D. of three independent experiments.

3.5. Monovalent cation-stimulated ATPase activity of TcENA

We sought first to measure the ATPase activity in the membrane fractions of transformed FreeStyle™ 293-F cells expressing TcENA-EGFP or EGFP (control) in the presence of 130 mM Na^+ and 20 mM K^+. To measure the monovalent cation-specific ATPase activity, calcium ATPases were inhibited by EGTA, and the contaminating activities of V-type ATPases and sarcoplasmic reticulum Ca^{2+}-ATPase were eliminated by the addition of bafilomycin and thapsigargin, respectively.

In the absence of orthovanadate, a specific inhibitor of P-type ATPases, we found the significantly higher ATPase activity in the membrane fraction of the TcENA-producing cells than that of the control ($P<0.05$, indicated by an asterisk in Fig. 5B), whereas overall ATPase activity of both membrane fractions was high. In the presence of orthovanadate, ATPase activity of both cells declined in a dose-dependent manner, and nearly 30% of the ATPase activity remained even in the presence of 50 μM orthovanadate, probably due to the contaminating non P-type ATPases in our membrane fractions (Fig. 5B). When the concentration of orthovanadate was ≥5 μM, there was no significant difference of ATPase activity between the experimental and control cells. These results indicate that TcENA possessed orthovanadate-sensitive ATPase activity, which is characteristic to a P-type ATPase, and that the ATPase activity of TcENA was stimulated by either Na^+ or K^+, or both cations.

We confirmed the presence of Na^+/K^+-ATPase activity, a plasma membrane marker, in our membrane fractions, in that addition of ouabain, a specific Na^+/K^+-ATPase inhibitor, drastically reduced the ATPase activity of TcENA-expressing and control cells (Fig. 5C). The residual ATPase activity (approximately 25 nmol/mg/min) of the control cells in the presence of ouabain (1 mM) was comparable to that in the presence of orthovanadate (≥30 μM), suggesting that the orthovanadate-sensitive ATPase activity of the control cells can be attributed to the native Na^+/K^+-ATPase. It is important to note that TcENA-expressing cells showed significantly higher ATPase activity than did control cells, both in the presence and in absence of ouabain ($P<0.01$, Fig. 5C). These results indicate that TcENA is an ouabain-insensitive, monovalent cation-specific ATPase.

Next, we addressed identification of the cation associated with TcENA ATPase activity, using a basal assay mixture containing 1 mM ouabain and 15 mM NH_4^+ instead of K^+ [23]. In this assay, the ionic strength was kept constant by adding choline chloride. The ATPase activity of the TcENA-expressing cells was significantly stimulated by either Na^+ or K^+ alone in a dose-dependent manner (Fig. 5D). Theses results clearly indicate that TcENA is stimulated not only by Na^+ but also

by K^+. At the lower concentrations of the monovalent cations (10 mM), we observed higher ATPase activity for K^+ than for Na^+, while the reason was not clear.

3.6. Tolerance to high Na^+ stress in TcENA-overexpressing *T. cruzi*

Using genetically modified epimastigotes, we sought to determine the physiological role of TcENA in *T. cruzi*. Epimastigotes were transfected with a trypanosomal expression plasmid, pTREX, carrying the *TcENA-EGFP* fusion gene. Expression of the TcENA-EGFP protein in the resulting transformants (TcENA-*T. cruzi*) was confirmed by Western blot and indirect fluorescence analyses using the anti-TcENA antibody (data not shown). In LIT medium, TcENA-*T. cruzi* displayed a slightly faster growth rate than the control cells, which had been transformed with pTREX alone (Fig. 6A).

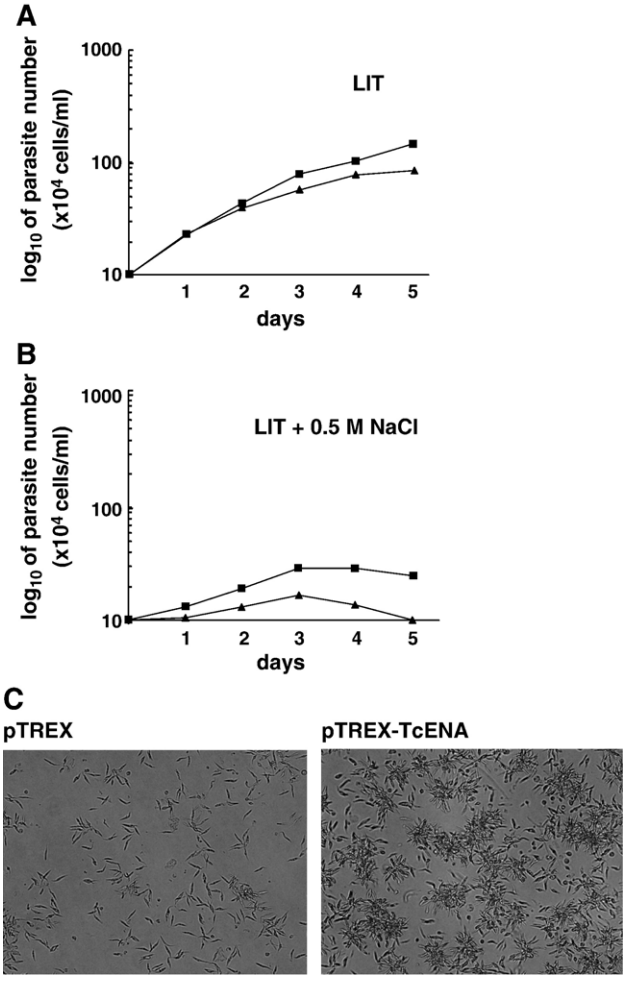

Fig. 6. Phenotypic analyses of TcENA-overexpressing *T. cruzi* epimastigotes under conditions of high Na^+ stress. Growth curve of TcENA-overexpressing (closed squares) and control (closed triangles) epimastigotes in the absence (A) or presence (B) of 0.5 M NaCl. Each data point represents the mean of quadruplicate determinations. (C) Microscopic observation of the TcENA-overexpressing (right) and control (left) epimastigotes. Note that epimastigotes of both groups attached to the bottom of the culture flask, but that the rosette-formation was only observed in TcENA-overexpressers. The video images are also available on line (Supplementary video).

We proceeded to test the susceptibility of TcENA-*T. cruzi* to high Na^+ stress. Both the TcENA-*T. cruzi* and control epimastigotes were cultured in LIT medium containing 500 mM NaCl, and parasite growth was monitored for 5 days. The parasite growth was gradually suppressed in both the experimental and control epimastigotes, most likely due to high Na^+ concentration in the medium (Fig. 6B). Interestingly, the number of TcENA-*T. cruzi* epimastigotes was significantly higher than that of control epimastigotes, indicating that overexpression of TcENA leads to tolerance to high Na^+ stress.

We found that the motility and behavioral patterns differed for TcENA-*T. cruzi* and control epimastigotes (Supplementary video is available on line). After 14 days in cultivation, control epimastigotes became less motile and attached individually to the bottom of the culture flask (Fig. 6C). In contrast, TcENA-epimastigotes showed intense movement and formed rosette-like aggregations, similar to those observed in normal cultivation. These findings indicate that overexpression of TcENA in *T. cruzi* confers salt tolerance on the parasite, which is most likely driven by accelerated Na^+ efflux.

4. Discussion

For many parasitic organisms, the crucial steps in their life cycle include the transmission between hosts and entry into their definitive habitats. These changed are accompanied by rapid and drastic changes in the environmental conditions of the parasite. Intracellular parasites, such as *T. cruzi*, especially require adaptation to different ionic environments. *T. cruzi* has a complex life cycle, which includes the obligatory mammalian stages in the host cell cytosol and in the blood stream. In these two environments, the parasite lives under conditions of extremely different Na^+ and K^+ balance.

In the present study, we identified a gene encoding Na^+-ATPase in *T. cruzi*, which we termed *TcENA*. The deduced amino acid sequence of TcENA showed significant similarity with those of fungal and plant ENA, which have been characterized as Na^+ and K^+ efflux pumps. A search of the *T. cruzi* genome sequence database revealed the presence of two *TcENA* genes, which share a 99.3% amino acid sequence identity. *T. cruzi* strain CL Brener, the reference strain in the *T. cruzi* genome project, has a hybrid genome comprised of two distantly related *T. cruzi* lineages [24]. The presence of two *TcENA* genes with sequence variations may reflect the hybrid nature of the CL Brener genome.

Previous analyses of fungal and plant ENAs have been limited to a survey of defective phenotypes in *ENA*-disrupted yeast mutants and to phenotypic complementation using heterologous expression [25,26]. Therefore, biochemical properties of ENAs, including their ATPase activities and the ionic stimulants, had not yet been determined. In the present study, we established a unique assay system for TcENA using a mammalian expression system and an established enzyme assay [15], since the plasma membrane fraction of *T. cruzi* contains high levels of H^+-ATPase activity, which would mask its Na^+-ATPase activity [27]. This system provides several advantages for analyses of ENA-type ATPases, since mammals lack ENA;

mammalian P-type ATPases have been well characterized; and inhibitors of various type of ATPases are available.

Using this assay system, we could demonstrate for the first time that TcENA possessed the orthovanadate-sensitive ATPase activity, which is characteristic to P-type ATPases. In the presence of ouabain, ATPase activity of the membrane fraction of TcENA-producing cell was significantly higher than that of the control cells, indicating that TcENA is an ouabain-insensitive ATPase. Furthermore, the TcENA-specific ATPase activity was stimulated in the presence of either Na^+ or K^+, dependent on the concentration of cations. Dependence of the ATPase activity of TcENA on the ion strength is unlikely as the ionic strength was kept constant. Since Na^+/K^+-ATPase does not function in the absence of either Na^+ or K^+, these results provide evidence that TcENA is actually expressed in the transformed cells and possesses the Na^+- or K^+-stimulated ATPase activity. On the other hand, our membrane fractions showed the high level of the background ATPase activities, probably due to the contaminating non P-type ATPases, such as membrane-associated, cytoskeletal actin. Further improvement of the assay system, especially for reducing the background ATPase activities, is necessary to determine the exact enzyme properties of TcENA.

Our biochemical analysis clearly showed that TcENA is an ouabain-insensitive and Na^+- or K^+-stimulated ATPase. The presence of ouabain-insensitive, Na^+-stimulated ATPase activity has been previously suggested in *T. cruzi*, but not been molecularly identified [11,27]. Thus, the latter activity can most likely be attributed to TcENA. An orthologous gene of *TcENA* is conserved in other trypanosomatids, including *L. donovani* and *T. brucei* [18], which are the pathogens for the important tropical diseases, Kala-azar and African sleeping sickness, respectively. The presence of an ENA-type ATPase in trypanosomatids and its absence from humans may provide insights into the trypanosomatid ENA-type ATPases as drug targets in the treatment of trypanosome infections.

Although *TcENA* is expressed throughout the *T. cruzi* life cycle, its transcription level was higher in epimastigotes and trypomastigotes than in amastigotes. These findings are in agreement with the facts that epimastigotes and trypomastigotes can live in conditions of high Na^+ concentrations. Trypomastigotes live in the blood stream, which contains 145 mM Na^+, and epimastigotes grow well in medium containing the same Na^+ concentration. In addition, we found that epimastigotes overexpressing TcENA are active in medium containing extremely high concentration of Na^+, strongly suggesting that TcENA functions as a sodium efflux pump and confers tolerance to high Na^+. Amastigotes, however, live in the host cell cytosol, where the concentration of Na^+ is much lower, suggesting that, in this stage, TcENA has less importance in Na^+ efflux. Thus, differences in transcription level between the extracellular and intracellular forms may reflect differences in environmental Na^+ concentration.

Similar to other ENAs, we found that TcENA is stimulated not only by Na^+ but also by K^+. Although the physiological importance of the ability of *T. cruzi* TcENA to extrude K^+ is unclear, it is possible to speculate that K^+ efflux is necessary for intracellular amastigotes. Amastigotes live in the host cell cytosol and are exposed to higher concentrations of K^+ than in the blood stream . It is noteworthy that amastigotes express K^+ channels, which allow import of K^+ across the plasma membrane [27]. Thus, TcENA may play dual roles in parasite development. In epimastigotes and trypomastigotes, it may function as a sodium pump, whereas in amastigotes, it may be involved in the maintenance of K^+ concentration. That is, K^+ homeostasis may be balanced by import via K^+ channels and export via TcENA.

Fungal ENAs are thought to have different preferences for Na^+ and K^+ [28]. For example, *Neurospora crassa* ENA appears to be associated with Na^+ efflux, whereas *S. pombe* ENA (cta3) seems to be a K^+-ATPase [28]. We found that TcENA displayed higher ATPase activity for K^+ than for Na^+ at the lower concentration of cations (10 mM). This may reflect a difference in transporting efficiency, while its physiological significance is unclear.

In addition to the ouabain-insensitive TcENA, the presence of an ouabain-sensitive sodium pump in *T. cruzi* has been suggested [27]. To determine whether the latter activity could be attributed to Na^+/K^+ ATPase, we extensively searched for an ortholog of mammalian Na^+/K^+ ATPase in the genome sequence databases of trypanosomatids, including *T. cruzi*, but we were unable to find any. Further analyses are necessary to understand the physiological roles of TcENA, using parasites lacking the *TcENA* gene, as well as the mechanism of regulation of Na^+ and K^+ homeostasis in *T. cruzi*.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbamem.2006.04.025.

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