Purification, Cloning, and Characterization of an Acidic Ectoprotein Phosphatase Differentially Expressed in the Infectious Bloodstream Form of Trypanosoma brucei*

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We purified an ecto-phosphatase of 115 kDa (TryAcP115) specifically expressed by bloodstream forms of Trypanosoma brucei. The corresponding gene coded for a 45-kDa protein potentially including a signal peptide, a membrane-spanning domain and an N-terminal domain containing 8 N-glycosylation sites. There was no significant sequence homology with other phosphatases. Antiserum to the Escherichia coli recombinant N-terminal domain, Petase7, recognized a protein of 55 kDa in Western blots after deglycosylation of the TryAcP115 protein by N-glycosidase F. Immunofluorescence and trypsin treatment of living parasites showed that TryAcP115 was localized to the surface of the parasite and that its N-terminal domain was oriented extracellularly. The recombinant N-terminal domains, expressed in E. coli and Leishmania amazonensis, harbored phosphatase activity against Tyr(P)-Raytide, Ser(P)-neurogranin, and ATP. The enzymatic properties of native TryAcP115 and the recombinant proteins for the substrate Tyr(P)-Raytide were virtually identical and included: (i) $K_m$ and $V_{max}$ values of 15 nM and 200 pmol/min/mg, (ii) no requirement for divalent cations, and (iii) sensitivity to vanadate, sodium fluoride, and tartrate, but insensitivity to okadaic acid and tetramisole. Although the function of TryAcP115 remains unknown, a differentially expressed, unique ecto-phosphatase could regulate growth or influence parasite-host interactions and might provide a useful target for chemotherapy.

Control levels of extracellular ATP and surface protein phosphorylation are important physiological regulatory mechanisms (1, 2), but the correlation between the two in the regulation of cellular function has yet to be fully established. Extracellular ATP levels are under the control of ecto-ATPases and ecto-apyrases, which can be distinguished by the substrates they hydrolyze, NTPs¹ and both NTPs and NDPs, respectively (3). These ecto-enzymes may be involved in major cellular processes including adhesion, termination of purigenic signaling, and purine recycling (2).

Reversible phosphorylation of protein extracellular domains is controlled by a novel class of protein kinases, called ecto-protein kinases. This extracellular catalytic activity has been identified at the surface of many cell types. Ecto-kinases either reside on (4, 5) or are shed from the cell surface (6–8). Ecto-phosphatase activities have also been detected in some mammalian cells (9–11) and in two parasitic protozoa Trypanosoma cruzi (12) and Trypanosoma brucei (13). Cellular activation, motility, growth, and differentiation are partially regulated by the combined action of ecto-kinases and ecto-phosphatases (6).

Because the proteins controlling extracellular phosphorylation and extracellular ATP levels are thought to play a central role in the modulation of cell growth (14, 15), T-cell activation (5, 10, 16), and parasite-host interaction (17, 18), identification of ecto kinases and phosphatases in a primitive parasite such as T. brucei would be of great interest. T. brucei is a digenetic parasitic protozoan with a complex life cycle characterized by proliferative and non-proliferative stages, accompanied by biochemical and morphological changes depending on the insect and the mammalian host (19). It also alternates between infectious and noninfectious stages, but the mechanisms that regulate these alternating periods of growth and infectivity are unknown. Immunosuppression, including a depressed T-cell response (20), is considered to be an essential element of the host-parasite relationships in the early stages of infection (21).

Previously we characterized differentially expressed, plasma membrane-associated tyrosine phosphatase activity in T. brucei (22). By monitoring tyrosine phosphatase activity, we have now purified and cloned a membrane protein phosphatase without significant sequence homology with other phosphatases. Kinetic values, substrate specificity, and modulator profiles were similar to those of acidic protein phosphatases. These characteristics and the extracellular localization of the catalytic domain suggest that this protein belongs to a new family of acidic ecto-phosphatases.

EXPERIMENTAL PROCEDURES

Trypanosomes and Leishmania—T. brucei brucei monomorphic clone MITat 1.4 (23, 24) and clone AnTat 1 (25) were harvested at mid-log phase from infected rats and isolated by DEAE-cellulose chromatography (26). Procyclics were transformed from the MITat 1.4 clone and

¹ The abbreviations used are: NTP, nucleotide triphosphate; MES, 4-morpholineethanesulfonic acid; Pipes, 1,4-piperazinediethanesulfonic acid; DTT, dithiothreitol; PnPP, p-nitrophenyl phosphate; RT-PCR, reverse transcriptase-polymerase chain reaction; PBS, phosphate-buffered saline; PTPase, phosphotyrosine phosphatase.

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cultivated to late log-phase in a modified minimum essential medium (27). *Leishmania amazonensis* PRO/F/BR/72/18141 (LV79) was cultured at 24 °C in RPMI 1640 without phenol red and supplemented with 25 mM Hepes, 2 mM glutamine, 2 mg/ml dextrose, 1 mM sodium pyruvate, 50 μg/ml streptomycin, minimal essential medium and non-essential amino acids, 10% fetal calf serum.

**Preparation of Membrane Fractions**—Purification from the total cell extract (F1) was performed as described by Seyfang and Duszenko (28). Briefly, F2 was a ghost fraction obtained by hypotonic lysis, F3 was the F2 fraction stripped of peripheral and cytoskeletal proteins by EDTA/alkali treatment, and F4 was the cytoplasmic-glucose-solubilized protein fraction. F5 was the TBS-consisted cytoplasmic-glucose-solubilized protein fraction obtained by treating F3 for 30 min at 4 °C with gentle agitation with 0.5% Triton X-100 and centrifugation for 1 h at 100,000 × g. Protein concentrations were determined by the method of Bradford (29), 0.1% Triton X-100 was used to solubilize the proteins when necessary. Bovine serum albumin was used as a standard.

**Columns and Instruments**—DEAE-Sepharose fast flow (Amersham Pharmacia Biotech, Saclay, France), was used for ion exchange chromatography. O-Phospho-L-Tyrosine (O-Tyr(P)) immobilized on cross-linked 4% beads agarose Sigma was used for affinity chromatography. The chromatographic system used throughout this study was the FPLC workstation from Amersham Pharmacia Biotech. All buffers contained a protease inhibitor mixture with final concentrations of 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, and 10 μM phenylmethylsulfonyl fluoride.

**Ion Exchange Chromatography**—The F3 fraction was equilibrated in buffer A1 (40 mM MES, pH 5.6, 0.5% Triton X-100, 20 mM NaCl, 1 mM EDTA, protease inhibitor mixture) and loaded on the DEAE fast flow column from Amersham Pharmacia Biotech pre-equilibrated in buffer A1. The column was then washed with buffer A1 and the breakthrough collected. Elution was performed by the discontinuous step gradient method. Fractions were collected at 70 and 100% buffer B1 (40 mM MES, pH 5.6, 0.5% Triton X-100, 200 mM NaCl, 1 mM EDTA, protease inhibitor mixture). Proteins were eluted at each step and the major phosphotyrosine phosphatase (PTPase) activity was identified at the 70% buffer B1 step. Flow rate was 2 ml/min for each step.

**Affinity Chromatography**—The 70% eluted DEAE fraction was diluted and equilibrated with pre-equilibration buffer A2 (20 mM sodium acetate, pH 4, 0.5% Triton X-100, 20 mM NaCl, 1 mM EDTA, protease inhibitor mixture) and adsorbed onto a buffer A2 pre-equilibrated O-Tyr(P)-agarose affinity column. The column was washed with buffer A2 and the breakthrough collected. The enzyme was eluted with a linear salt gradient from 20 to 500 mM NaCl in buffer B2 (50 mM sodium acetate, pH 4, 0.5% Triton X-100, 200 mM NaCl, 1 mM EDTA, protease inhibitor mixture) in a total volume of 20 ml. A major protein peak with PTPase activity was eluted at 150 mM salt. Flow rate was 1 ml/min for each step. Petase7, the recombinant protein expressed in *E. coli* was phosphorylated with pKa (Promega) and [γ-32P]ATP (34 000 cpm/pmol), ATPase activity was followed by measuring the release of phosphate according to Mitsui et al. (31) at pH 6.9 using 1 mM [γ-32P]ATP (55,000 cpm/nmol). Before the assay, protein extract buffer was exchanged by PEM buffer (0.1% Pipes, 1 mM MgCl2, 1 mM EGTA, pH 6.9) using a PD10 column from Amersham Pharmacia Biotech.

**Microsequencing**—Germline microsequencing was performed from an F2 fraction Black-stained SDS-polyacrylamide gels and sent for microsequencing to the "Laboratoire de Microsequençage des Protéines" Dr. Dalayer, Institut Pasteur Paris (32).

**Cloning by Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) and Analysis of the tryAcP115 Gene**—Total cell RNA and poly(A)+ RNA were prepared and purified according to Sambrook et al. (33). Oligo-dT primers containing with Anxamucleotides and reverse transcription with the Stratagene reverse transcriptase RT II. The P1CaS (5'-ATNACYTGGTNCACCAARTA-3'), P3CaS (5'-TTRCTYTTCCACCTTCTC-3'), and P1CaS (5'-TATYTYGGNAGY-CARGTNAT-3') degenerate oligonucleotides were generated by the reverse translation of sequenced peptides, P1 (YFGSQVQ) and P3 (DEWESK) and translation of P1 (YFGSQVQ), respectively. A portion of the common splice leader sequence of *T. brucei* mRNAs (5'-ACAGTT-TCTGACTATATGTG-3') was also used as a 5' primer (ME2X). The cDNA was then used as a template for PCR amplification using the primer associations MEX2/P3CaAS, MEX2/P1CaAS, and P3CaS/P1CaS. A 5' fragment of 5000 base pairs was isolated and sequenced using the AmpliTaq DNA polymerase, as described by the manufacturer (ABI PRISM™, Perkin Elmer).

**Production and Western blot Analysis of Immune Serum against the Petase7 Protein**—A rabbit was injected with 150 μg of Petase7 in complete Freund's adjuvant and with 100 μg in incomplete 15 and 30 days later. Blood was collected before the first injection (preimmune serum) and 10, 13, and 15 days following the last injection. Affinity purified proteins in sample buffer (2.2% SDS, 50 mM DTT, 80 mM Tris-HCl, pH 6.8, and 10% glycerol, mass/volume) were boiled for 5 min and subjected to 10% polyacrylamide gel electrophoresis. Proteins were transferred to polyvinylidene difluoride (Immobilon P, Millipore) membranes by semi-dry blotting (36). Filters were blocked for 15 min with PBS/Tween/milk (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na2HPO4, 1.4 mM KH2PO4, pH 7.4) and 5% milk in PBS/Tween/milk. The membranes were incubated for 1 h at room temperature with the fluorescein isothiocyanate-labeled secondary antibody (Pasteur Laboratories). Binding bands were revealed by washing in PBS/Tween/milk or 1/2000 dilution of anti-rabbit IgG conjugated to horseradish peroxidase (Sanofi-Pasteur) in PBS/Tween/milk or 1/2000 goat anti-rabbit IgG conjugated to alkaline phosphatase (Bio-Rad) in PBS/Tween/milk. Immunoreactive bands were revealed by washing in 50 mM Tris-HCl, pH 7.5, 20 mM NaCl and a solution containing 0.5% H2O2, 100 mM PMSF, 5.4 mg/ml chemiluminescent substrate, and aspirated to the membrane. Membranes were exposed to Amido-Black-stained SDS-polyacrylamide gels and sent for microsequencing to the "Laboratoire de Microsequençage des Protéines" Dr. Dalayer, Institut Pasteur Paris (32).

The amplified and cloned MEX2/P3CaAS fragment was used as a probe to screen a *T. brucei* genomic DNA library (33) generated into the cZX75 cosm id vector (34) as described previously (35). KpnI fragments of the isolated L3 cosm id were subcloned in the pUC18 vector from Appligne and screened with the cDNA fragment MEX2/P3CaAS. A KpnI fragment of 5000 base pairs was isolated and sequenced using the AmpliTaq DNA polymerase, as described by the manufacturer (ABI PRISM™, Perkin Elmer).

**Immunofluorescence**—Bloodstream form trypanosomes (AnTat) were obtained at peak parasitemia from infected mice, and fixed on ice for 2 min in 0.2% formaldehyde. The formaldehyde was neutralized with 10 min with 0.1 μg/ml of 4-vinylpyridine, at room temperature. After centrifugation and resuspension in PBS, trypanosomes were transfused to a microscope slide and treated with anti-Petase7 diluted (1/500) in PBS containing 0.1% bovine serum albumin for 30 min at room temperature followed by the fluorescein isothiocyanate-labeled secondary antibody (Pasteur Sanofi) diluted (1/1000) in PBS, 0.1% bovine serum albumin, 0.001% Evans blue for an additional 30 min. After washing, slides containing the treated trypanosomes were mounted with anti-fade Vectashield (Vector Laboratories).

For *Leishmania*, 10⁵ promastigotes were transfused to circular glass poly-t-lysine-treated (30 min. in 10 μg/ml) coverslips and placed inside a 24-well plate. After centrifugation at 130 × g for 10 min, cells were fixed with 2% paraformaldehyde for 1 h and washed twice with PBS before fixation for 1 h at room temperature with anti-Petase7 serum, followed by 1000-fold in PBS containing 10% mouse serum. After three washes of 10 min each in PBS, fluorescein-conjugated goat anti-rabbit IgG diluted 2000-fold in PBS with 10% goat serum was added as a secondary antibody. Photomicrographs were taken through a Zeiss UV microscope and images analyzed by the use of a camera (Photometrics) with IPLab software (Sigma Analytics) and Adobe photoshop 4.0 on a Macintosh 7100/90 computer.
Cloning, Expression, and Purification of the N-terminal Domain of TryAcP115 in E. coli—A 1-kilobase fragment comprising the N-terminal region lacking the putative signal peptide was generated by PCR. A 5-kilobase KpnI genomic fragment from AnTat 1 subcloned in pBlue-Script (Stratagene) was used as a template in the PCR reaction. The 5’ primer (5’-GGCAAAACATATGGATCGAGCGATGCCGCAA-3’) contained a 12-nucleotide linker with a NdeI restriction site to facilitate subcloning and 7 adjacent N-terminal residues (ESSSSSDD). The 3’ primer, (5’-ACAAGGATCTTACTGATGCTGATGCTGATGCTCGGGATCCTTACTGATGCTGATGCTCGGGTGATGCGCAA-3’), included a 12-nucleotide linker with a BamHI restriction site to facilitate cloning, a stop codon, codons for 6-histidine tag residues (PLTIPE) and an NdeI restriction site to facilitate subcloning and 7 adjacent N-terminal residues (ESSSSSDD). The resulting recombinant gene coding for the mutated protein PetaseM4 was obtained by using the site-directed mutagenesis kit from CLONTECH, Petase7 as DNA matrix and the following mutated oligonucleotides MUT1 (ACCGGCACTCCGGAGAAGTCAAG) and MUT2 (GAGTCAACACGGCGACACTGATGT). The resulting recombinant Petase7 and PetM4 proteins were expressed in E. coli BL21 (DE3) from Novagen according to the manufacturer’s instructions. Cells were lysed in 1× binding buffer, containing the protease inhibitor mixture, by three steps of freezing and thawing and brief sonication. The lysate was centrifuged for 30 min at 10,000 × g and the resulting supernatant applied to a Ni²⁺-His-Bind™ column.

Cloning, Expression, and Purification of the N-terminal Domain of TryAcP115 in Leishmania—To ensure the presence of the signal peptide, the N-terminal region was PCR amplified with the following primers designed with the same characteristics as for E. coli: L115PSNter (CTAGCCGGGGAATTCATCCTGCGGACTATGATG) and L115PSer (CCG-CAGTCAATGATGCTGATGCTGATGCTCGGGTATCGTTAACGG). The fragment was cloned in the pTEX vector (gift of Dr. J. Kelly (37)) at EcoRI/BamHI sites giving the pTEX/AcP construction. 10⁶ exponentially growing cells (L. amazonensis LV79) were washed and resuspended in 200 µl of cold electroporation buffer (21 mM Hepes, pH 7.4, 137 mM NaCl, 5 mM KCl, 0.7 mM NaH₂PO₄, 6 mM glucose). 50 µg of plasmid DNA was added and the cells electroporated at 0 °C in 2-mm cuvettes with a Cellject apparatus set on 450 V, 74 W, 600 microfarads, single pulse mode. Cells were transferred to 10 ml of culture medium and 24 h later G418 (10 µg/ml) was added. For purification, 50 ml of the culture supernatant was applied to a 50-ml Q fast flow column on a 1× binding buffer (20 mM Tris, pH 7.9, 500 mM NaCl, 6 mM imidazole). The nonretained fraction was applied to the His-Bind™ column and the pTEX/AcP protein eluted as described above.

RESULTS

Purification of the 115-kDa Acidic Phosphatase (TryAcP115) from T. brucei—Since the detergent n-octylthioglucoside used in our earlier study (30) solubilized, but inactivated, the phosphatase activity, we tested the effect of 0.5% Triton X-100. The resulting PTPase activity in the Triton X-100-solubilized fraction (F4TS) shared the characteristics of the original TbPTPase activity of the F4 membrane fraction (30). It was sensitive to 10 nM vanadate and 1 mM sodium fluoride, was more active at pH 4 than pH 7, and had a K_m value of 10 nM for Tyr(P)-Raytide. Next we devised a two-step chromatographic purification procedure (Fig. 1). Chromatography on DEAE fast flow at
pH 5.6 (Fig. 1, A and B) removed VSG (60 kDa), the major surface protein of the F4TS fraction and two other abundant proteins of 70 and 30 kDa (Fig. 1B). Dephosphorylating activities were recovered (Table I) both in the breakthrough (BT-DEAE) and in the 150 mM NaCl elute (150EL-DEAE). The specific activity of the 150EL-DEAE on Tyr(P)-Raytide was 32 (±18) pmol/min/mg, which represented a purification of 14 (±4)-fold with a yield of 89% (±10). Purification of the DEAE eluted activity (Fig. 1C) on an O-phospho-l-tyrosine affinity column resulted in a large increase in the tyrosine phosphatase specific activity of the eluate (400EL-AFF) to 206 (±10) pmol/ min/mg, which represented a total purification factor of 90 (±20)-fold and a yield of 44% (±20) (Table I). The values of the purification parameters were similar when measured by either a specific (Tyr(P)-Raytide), or a nonspecific (pNPP) substrate, suggesting that the isolated activity dephosphorylated both Tyr(P)-Raytide and pNPP. The optimum activity of the affinity eluted fraction for Tyr(P)-Raytide was at pH 4. Affinity eluted PTPase activity catalyzed the removal of phosphate from Tyr(P)-Raytide with a $K_m$ value of 15 nM. These values (not shown) were similar to those estimated for F4 and F4TS. Examination of the 400EL-AFF fraction by SDS-PAGE under reducing conditions and by Coomassie staining (Fig. 1D) showed the presence of a major polypeptide of 115-kDa and two minor polypeptides of 66 and 30 kDa. The 30-kDa polypeptide was not well stained by Coomassie. Protein-reconstitution assays (Fig. 1E) indicated that the PTPase activity was in the 115-kDa polypeptide, which we named 115 acidic phosphatase protein (TryAcP115).

**Sequencing and Cloning of TryAcP115**—5 μg of TryAcP115 protein were obtained from 1.5 × 10^{11} bloodstream form trypanosomes. The protein was further purified by 10% reducing SDS-PAGE. Microsequencing of peptides generated by Endo-K cleavage and high performance liquid chromatography purification yielded 2 peptide sequences: 1) RGVY-FGSQVISLSGR; and 2) KAFDEWESK (Fig. 2A). Degenerated primers and minixenor primers were designed according to the sequence alignments with Match-Box and Bisance service (40) and sequence alignments with Match-Box service (41) revealed no protein with statistically significant similarities to the tryAcP115 gene sequence.

**Genomic Southern Blot and mRNA Expression Analysis of T. brucei AnTat tryAcP115 Gene**—After digestion of genomic DNA with several restriction enzymes, Southern blot patterns indicated (data not shown) that these trypanosomal genomes harbored a single copy gene of tryAcP115.

**Expression, Glycosylation, and Localization of the TryAcP115 Protein**—An histidine-tagged recombinant protein, Petase', containing the N-terminal part of TryAcP115 deleted from the signal peptide (Fig. 2A) was expressed in E. coli, purified by Ni^{2+}-His-TrapTM and affinity O-Tyr(P)-agarose chromatography and used to immunize rabbits. Western blots confirmed the differential expression of the 115 acidic protein phosphatase. It (Fig. 3A) was recognized by the rabbit antiserum in fraction F3 of bloodstream trypanosomes, but not in the equivalent procyclic trypanosome fraction. Several questions raised by the sequence analysis including the degree of glycosylation, the membrane localization, and the orientation of TryAcP115 were also addressed. The antiseraum reacted in immunoblots with a high molecular mass band of 115-kDa (Fig. 3, A and B) which shifted to 55-kDa after N-glycosidase F treatment (Fig. 3B, lane F4 dg) showing that the TryAcP115 protein (45.3 kDa estimated molecular mass) was highly glycosylated. A minor band of about 66 kDa (Fig. 3B, lanes F2, F3, and F4) was also recognized and may represent a partially glycosylated product of TryAcP115 or cleaved peptide. Enrichment of the TryAcP115 protein content during membrane fraction preparation was accompanied by enrichment of total plasma membrane protein in the successive fractionation steps (Fig. 3B, lanes F2, F3, and F4) (30). Under these conditions TryAcP115 was not detectable in the total cell lysate fraction (F1).
Indirect immunofluorescence showed that the antiserum reacted specifically with surface elements of the parasite body (Fig. 4). Interestingly, the protein appeared to be unequally distributed along the cell body. Regions with higher fluorescence density indicated a patchwork organization of small protein aggregates and larger fluorescent regions. Subsequently, we attempted to determine the orientation of TryAcP115 across the membrane. Although the N terminus was glycosylated (Fig. 3B) and a signal peptide had been observed (Fig. 2), we had no definitive evidence that the N-terminal domain was extracellular. In order to study this orientation further, we digested surface proteins of living trypanosomes with trypsin for 5, 10, and 15 min (Fig. 3C). During the treatment and 1 h after blocking, the motility of the cells was unaffected, and the parasites retained their elongated shape. Analysis of these proteins by SDS-PAGE, Coomassie staining, and Western blotting with the same rabbit antiserum showed that a 30-kDa peptide was released from the TryAcP115 protein (Fig. 3C). Control trypanosomes incubated without trypsin (Fig. 3C) released neither the TryAcP115 protein nor the corresponding peptides.

**Isolation and Kinetic Parameters of Wild Type Petase7, Mutant PetM4, and pTEX/AcP Proteins**—For further verification...
that the tryAcP115 gene encoded a protein with intrinsic PT-Pase activity, we examined the recombinant proteins Petase7 and PTEx/AcP (entire N-terminal domain expressed in Leishmania) for phosphatase activity (Fig. 5). Petase7 overexpression and purification by a two-step procedure were analyzed by Coomassie staining (Fig. 5A). PTEx/AcP expression was examined by immunofluorescence (Fig. 5, D and E). A 60-kDa protein in the culture supernatant of Leishmania was identified, after immobilized metal affinity chromatography purification, by probing Western blots with the Petase7 antiserum (Fig. 5B). Phosphatase activity against pNPP was undetectable in the corresponding purified fractions of E. coli and Leishmania controls expressing non-related proteins. Tyr(P)-Raytide was dephosphorylated by Petase7 with an optimum pH of 4 (Fig. 5B). The specific activity was estimated to be 3.6 (±2.6) pmol/min/mg. However, the specific activity of PTEx/AcP against the same substrate (200 pmol/min/mg) was much higher (Fig. 5G). This large difference in specific activity could be partially due to the presence of protein inactivated by improper folding in E. coli. As shown in Fig. 5 (see legend), only 5–20% of the His-Tag eluted protein was retained on the O-Tyr(P) affinity column. Although the specific activity of Petase7 for Tyr(P)-Raytide increased after this purification step, it only reached an average value of 16 (±13) pmol/min/mg. Interestingly, the specific activity of PetaseM4, a mutated Petase7 enzyme, with Cys-166 and -185 replaced by serine and glycine residues, respectively, was reduced to 0.2 (±0.1). These cysteine residues were initially chosen because they were part (the arginine residue was absent) of the hallmark of the PTPase family (42). In contrast, the $K_m$ values of Petase7 and PTEx/AcP of about 15 nM were similar to tryAcP115 (Fig. 5, C and G).

Inhibitor Profiles of Petase7 Protein—By protein reconstitution (30) we have previously characterized a 115-kDa peptide in the F4 fraction as a protein-tyrosine phosphatase sensitive to vanadate, tartrate, and sodium fluoride. A modulator profile similar to the F4TS fraction was obtained with tryAcP115, Petase7, and PTEx/AcP. Among the usual phosphatase inhibitors tested, vanadate, NPP(phen), molybdate, sodium fluoride, and tartrate inhibited the phosphatase activity with IC$_{50}$ values of 3, 2, 4, 100, and 250 μM, respectively. None of these preparations were affected by 150 mM okadaic acid, an inhibitor of type-2A and type 1 protein Ser/Thr phosphatases, nor by 1 mM tetranirole, which inhibits alkaline phosphatases. The divalent cations, Ca$^{2+}$, Cu$^{2+}$, Zn$^{2+}$, and Ni$^{2+}$ tested at 1 mM inhibited the phosphatase activity by about 50% while Mg$^{2+}$ stimulated it (Table II).

To test the substrate of TryAcP115 and the recombinant proteins, we first examined three sets of phosphorylated substrates (phosphorylated nucleotides, phosphorylated sugars, and tyrosine phosphatase substrates) as inhibitors of Petase7 phosphotyrosyl phosphatase activity. ATP at only 1 mM inhibited significantly (50%), while 5 mM pNPP were necessary to inhibit equally. The serine-phosphorylated substrate, Kemptide, was a poor inhibitor (20% at 1 mM). Other phosphorylated sugars, O-phospho-L-tyrosine, and other nucleotides failed to inhibit significantly. In a second step, compounds which inhibited the phosphotyrosyl phosphatase activity of TryAcP115 and the recombinant proteins were tested as substrates for their phosphatase activity. We used the PTEx/AcP protein in these assays because it exhibited the highest specific activity. As shown in Table III, while Ser(P)-Kemptide was not a substrate for PTEx/AcP, the recombinant enzyme dephosphorylated the nonspecific substrate pNPP, ATP, and the serine-phosphorylated peptide, neurogranin, with $K_m$ and $V_{max}$ values, respectively, of 26 mM, 1.8 μmol/min/mg; 250 μmol/min/mg; and 200 nM, 200 pmol/min/mg, respectively. In contrast, pNPP and Ser(P)-neurogranin were poor substrates for Petase7, but it dephosphorylated ATP with kinetic constant values of 200 μM and 24 nmol/min/mg for $K_m$ and $V_{max}$, respectively. This ATPase activity was magnesium dependent and inhibited by 1 mM azide, 5 mM ADP, and 1 mM NTPs at 50, 80, and 50%, respectively.

**DISCUSSION**

We have purified, cloned, and characterized an acidic ectophosphatase. Throughout the purification process the phosphotyrosyl peptide phosphatase activity and pNPP dephosphorylation cofractionated with a constant relative activity ratio (Table I). This tight association of phosphatase activities suggested that they were mediated by the same enzyme. Because the O-phospho-L-tyrosine ligand was a poor substrate for TryAcP115 (Table II), the O-Tyr(P)-agarose column retained the phosphatase without significantly dephosphorylating it. After the affinity purified active fraction was eluted with a salt gradient (Fig. 1), Coomassie and silver stains revealed three polypeptides of 115, 66, and 30 kDa. The 115-kDa polypeptide was identified by membrane reconstitution activity as the protein harboring the tyrosine phosphatase activity (Fig. LE). The 30-kDa protein and its genomic sequence were not homologous with known phosphatases. The 66-kDa protein was recognized by the antiserum to the N-terminal region of TryAcP115 suggesting that it was differently processed or partially degraded (Fig. 3B). The recombinant PTEx/AcP protein of 60-kDa expressed in *L. amazonensis* may correspond to this 66-kDa protein.

Sequence analysis of several *T. brucei* subspecies revealed that *tryAcP115* is invariant and ubiquitous in the *Trypanozoon* subgenus. Discrepancy between the apparent (115 kDa) and predicted (45 kDa) molecular mass, as well as the apparent mass (55 kDa) after N-glycosidase F (Fig. 3D) treatment confirmed modification by N-linked glycans. The existence of 8 potential sites of N-glycosylation within the N-terminal domain (Fig. 2A) corroborated this conclusion. The calculated isoelectric point of 5.62 may explain the slower migration on SDS gels of the deglycosylated protein and the expressed protein in *E. coli* (Petase7) (Fig. 3B).

The primary sequence of TryAcP115 predicted the presence of a N-terminal signal peptide, a large extracellular N-terminal domain, a C-terminal transmembrane domain, and a short C-terminal intracellular domain of 30 amino acids (Fig. 2). The N-terminal extremity contains the three structural features characteristic of a signal peptide (43, 44): (i) a positively charged N-terminal amino acid (n region); (ii) a central hydrophobic domain (h region) of 16 amino acids (7 residues is the...
minimum required); and (iii) a COOH-terminal polar domain containing a glycine residue (c region). This c region is about 4 to 6 amino acids long and typically contains a proline or a glycine residue. During translocation the signal peptide is cleaved by signal peptidase with specificity due partly to the structural features of the signal peptide. The sequence homology of TryAcP115 with signal peptides of RPTK of the ephrin family (Fig. 2B), may suggest cleavage by a similar endopeptidase (45). Although the recombinant L. amazonensis protein pTEX/AcP, containing this potential signal peptide was purified from the culture supernatant, it failed to identify similarities of the peptidase similar to that of the ephrin family, since the same protein deleted of the signal peptide was also recovered from the supernatant (data not shown).

In order to study the localization, membrane orientation, and phosphatase activity of the TryAcP115 protein, its N-terminal domain was cloned and expressed in E. coli. The recombinant protein, Petase7, was purified by His-Tag and affinity columns (Fig. 5A). Indirect immunofluorescence with antiserum to Petase7 confirmed the surface localization of the TryAcP115 protein (Fig. 4). It was unequally spread over the cell surface and organized in patchwork patterns of larger stained areas.

The extracellular location of its N-terminal region was verified when Western biots of trypsin-treated living trypanosomes (Fig. 3C) revealed a partially degraded N-terminal domain. Since no TryAcP115 protein was released without trypsin treatment, the presence of the soluble degraded protein in the Western blot is not due to secretion. This result strengthened our conclusion that the N-terminal domain of TryAcP115 protein was oriented extracellularly. The short C-terminal cytoplasmic tail was not homologous to any other intracellular domain. Furthermore, it contained neither a tyrosine residue nor a Leu-Ile motif, which are essential for endocytosis and lysosome targeting (46, 47). The importance of C-terminal domains in protein localization suggests that the intracellular domain of TryAcP115 may have a pivotal role in the distribution of this protein over the parasite body, but its lack of homology with other intracellular domains prohibits further speculation about its function.

To determine if the phosphatase activities demonstrated by the F4 and F4TS fractions and by the affinity eluted TryAcP115, Petase7, and pTEX/AcP proteins were due to the same protein, we compared their kinetic constants, modulator effect profiles, and substrate specificity. First, we observed that the
The phosphatase activity of petase7 protein was assayed against 100 nM Tyr(P)-Raytide or 50 mM pNPP.

### Table II

| Effectors | [Effectors] | Relative activity, Tyr(P)-Raytide | IC₅₀ |
|-----------|-------------|----------------------------------|------|
| Phosphatase modulator compounds | | | |
| Vanadate | 0.2 | 2 | 3 |
| bpV(phen) | 0.2 | 1 | 2 |
| Molybdate | 0.2 | 5 | 4 |
| Sodium fluoride | 1 | 3 | 100 |
| Tartrate | 1 | 10 | 250 |
| Tetramizol | 1 | 100 |
| Okadaic acid | 0.15 × 10⁻³ | 100 |
| PP | 1 | 100 |
| EDTA | 1 | 100 |

| Bivalent metal ions | | |
|---------------------|---|---|
| MgCl₂ | 1 | 180 |
| CaCl₂ | 1 | 60 |
| MnCl₂ | 1 | 100 |
| ZnCl₂ | 1 | 50 |
| CuCl₂ | 1 | 80 |
| NiSO₄ | 1 | 40 |

| Phosphonucleotides | | |
|---------------------|---|---|
| 5'-ATP | 1 | 50 |
| 5'-CMP | 1 | 85 |
| 3'-AMP | 1 | 80 |
| 3',5'-AMPc | 1 | 80 |

| Tyrosine phosphatase substrates | | |
|-------------------------------|---|---|
| Ser(P)-Kemptide | 1 | 80 |
| pNPP | 5 | 50 |
| Tyr(P) | 5 | 100 |

| Other phosphatase substrates | | |
|-------------------------------|---|---|
| Glucose 6-phosphate | 1 | 90 |
| Fructose 1,6-diphosphate | 1 | 80 |
| myo-Inositol 1,4,5-trisphosphate | 1 | 80 |
| Glycerol 3-phosphate | 1 | 100 |
| 2,3-Diphosphoglyceric acid | 1 | 100 |

The phosphatase activity of the pTEX/AcP protein was assayed against 100 nM Tyr(P)-Raytide, 50 mM pNPP, 500 nM Ser(P)-Kemptide, 1.5 mM Thr(P)-neurogranin, and 2 mM ATP.

### Table III

| Substrate | kcat | Km | kcat/Km |
|-----------|-----|----|---------|
| pNPP | 1.66 | 26 × 10⁻³ | 64 |
| Tyr(P)-Raytide | 1.7 × 10⁻⁴ | 15 × 10⁻⁹ | 11,300 |
| Ser(P)-neurogranin | 2.7 × 10⁻⁴ | 200 × 10⁻⁹ | 1,350 |
| Ser(P)-Kemptide | 0.14 | 250 × 10⁻⁶ | 560 |

* No detectable activity.

The phosphatase activity of TryAcP115 was assayed against 100 nM Tyr(P)-Raytide, 50 mM pNPP, 500 nM Ser(P)-Kemptide, 1.5 mM Thr(P)-neurogranin, and 2 mM ATP.

The phosphatase activity of the pTEX/AcP protein was assayed against 100 nM Tyr(P)-Raytide, 50 mM pNPP, 500 nM Ser(P)-Kemptide, 1.5 mM Thr(P)-neurogranin, and 2 mM ATP.

The phosphatase activity of petase7 protein was assayed against 100 nM Tyr(P)-Raytide or 50 mM pNPP.

Finally, we observed that the effect of modulators on TryAcP115, Petase7, and pTEX/AcP corresponded to those previously identified for the 115-kDa protein-tyrosine phosphatase (30): sensitivity to vanadate, sodium fluoride, molybdate, and tartrate, but resistance to tetramizole and okadaic acid. Attempts to classify TryAcP115 among phosphatases by analyzing its biochemical properties revealed five characteristics of tartrate-resistant acid phosphatase such as human prostatic acid phosphatase: (i) high affinity for phosphotyrosine substrates (Km = 10 nM), (ii) greater affinity for phosphotyrosine than phosphoserine peptides, (iii) low Vmax for phosphopeptides, (iv) little reduction of phosphate hydrolysis when associated with either Ser(P) or Tyr(P) (kcat = 2 × 10⁻⁴ s⁻¹), and (v) sensitivity to sodium fluoride, vanadate, and tartrate, but partial resistance to divalent cations such as Zn²⁺. These characteristics are in agreement with the reported acid phosphatase activities located on the surface of blood-stream forms of Trypanosoma congolense (49) and associated with surface membrane-containing fractions of Trypanosoma rhodesiense (50). The low optimum pH of TryAcP115 for Tyr(P)-Raytide and the surface location of acid phosphatases on these three trypanosomes could represent substrate specificity or indicate that they must function in an acidic environment.

To explore the substrate specificity of the phosphatase activity of TryAcP115, we determined the kinetic constant values of pTEX/AcP for ATP Tyr(P)-Raytide and Ser(P)-neurogranin. We used the kcat/Km parameter (Table III) to determine substrate quality since it involves all the steps from binding through the release of the phosphate product. The tyrosine-phosphorolated peptide, Raytide, yielded the highest value of kcat/Km ratio of 11,300 M⁻¹ s⁻¹. For comparison, the kcat/Km values for ATP and Ser(P)-neurogranin were, respectively, 1350 and 560 M⁻¹ s⁻¹. Although Tyr(P)-Raytide appeared to be the best substrate, the kcat/Km parameters do not exclude the possibility that TryAcP115 may contain ecto-ATPase activity. Since it was inhibited by NTPs and ADP, it is likely that it hydrolyzes all nucleoside triphosphates and ADP. Diphosphohydrolyase activity inhibited by azide are characteristics of the ecto-apyrase family.

Northern and Western (Fig. 3A) blots showed that the acidic phosphatase was life cycle regulated in T. brucei. It was expressed in bloodstream forms, but not in the insect procyclic form. Our data indicate that T. brucei TryAcP115 is an ecto-protein phosphatase without homology to other known phosphatases, suggesting that it may represent a new class of phosphatase. Although we do not know its biological function, ectophosphorylation regulation in ectodomains of functionally important surface proteins and/or soluble external substrates has been implicated in processes such as inhibition of cell growth (14) and parasite host interactions (51). We plan to address the question of whether the in vivo substrate of this unique phosphatase is an endogenous parasite protein, a host protein, or an external substrate such as ATP. Its surface accessibility, lack of homology to mammalian proteins, and possible involvement in essential cellular functions suggests that the TryAcP115 protein might provide an attractive target for chemotherapy.

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Purification, Cloning, and Characterization of an Acidic Ectoprotein Phosphatase Differentially Expressed in the Infectious Bloodstream Form of *Trypanosoma brucei*

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