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Purification and Characterization of a Human Recombinant T-Cell Protein-Tyrosine-Phosphatase from a Baculovirus Expression System†

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ABSTRACT: A 48-kDa human T-cell protein-tyrosine-phosphatase (TC.PTPase) and a truncated form missing an 11-kDa C-terminal segment (TCΔC11.PTPase) were expressed by using the baculovirus system and characterized after extensive purification. The full-length PTPase was restricted to the particulate fraction of the cells from which it could be released by a combination of salt and detergent. The enzyme was entirely specific for phosphotyrosine residues. It displayed a low level of activity toward phosphorylated, reduced, carboxamidomethylated, and maleylated lysozyme (RCML), but was 12 times more active toward phosphorylated myelin basic protein (MBP). By contrast, the 37-kDa form localized in the soluble fraction, and its activity toward RCML was 5 times higher than that observed with MBP. The autophosphorylated cytoplasmic domain of the EGF receptor served as substrate for both enzymes. Limited proteolysis of either protein gave rise to a 33-kDa fragment displaying the substrate specificity of the truncated form. These data lend further support to the view that the C-terminal segment of the T-cell PTPase serves a regulatory function, playing an important role in the localization and substrate specificity of the enzyme.

Phosphorylation of proteins on tyrosyl residues has been implicated in signal transduction and the control of cell growth, proliferation, differentiation, and transformation (Hunter & Cooper, 1985; Yarden & Ullrich, 1988). Obviously, this process is regulated by the interplay of numerous protein-tyrosine-kinases and -phosphatases [reviewed in Tonks and Charbonneau (1989) and Hunter (1989)]. A protein-tyrosine-phosphatase (PTPase 1B) of ca. 35 kDa was previously purified to homogeneity from human placenta and characterized (Tonks et al., 1988a,b). PTPase 1B was cloned from human placenta cDNA libraries (Brown-Shimer et al., 1990; Chernoff et al., 1990); the open reading frame predicts a protein of 50 kDa, indicating that the 35-kDa enzyme isolated was in fact a truncated form of the original molecule. Related PTPases of ca. 50 kDa were cloned from human T-cells (Cool et al., 1989) and rat brain (Guan et al., 1990), and a growing number of such molecules are being identified [for a review, see Fischer et al. (1991)]. The leukocyte common antigen, CD 45 [reviewed by Thomas (1989)], was the first transmembrane molecule found to possess PTPase activity (Charbonneau et al., 1988, 1989; Tonks et al., 1988c, 1990). Since then, a number of receptor-linked PTPases displaying a wide variety of external domains were cloned (Streuli et al., 1988; Krueger et al., 1990; Kaplan et al., 1990; Mathews et al., 1990; Sap et al., 1990). These enzymes contain two tandem domains in their intracellular segments, each homologous to the low molecular weight PTPases (Charbonneau et al., 1989).

The T-cell PTPase was recently expressed in baby hamster kidney (BHK)[1] cells and its function investigated. In contrast

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to isolated PTPase 1B which displayed a high specific activity of ca. 40 000 units/mg toward RCML, the T-cell enzyme was virtually inactive toward this substrate unless subjected to prior limited trypsinolysis. It was not clear whether this low activity was an intrinsic property of the enzyme itself or due to its interaction with inhibitory molecules (Cool et al., 1990). Therefore, in order to characterize the T-cell enzyme in vitro, the protein was purified from a high-level baculovirus expression system. This approach has been used successfully to express a number of eukaryotic proteins [reviewed in Luckow and Summers (1988)]; expression is driven by the strong viral polyhedrin promoter. The T-cell PTPase differs mainly from purified placenta PTPase 1B in that it has an additional 98 residues at the C-terminus. To address the function of this 11-kDa segment, a truncated enzyme (TCΔC11.PTPase) has previously been generated by inserting a stop codon after Arg-317 (Cool et al., 1990).

In this paper, we describe the expression, purification, and enzymatic properties of the full-length and truncated forms of the T-cells PTPase.

Materials and Methods

Restriction and modifying enzymes were supplied by Stratagene (LaJolla, CA), BRL (Gaithersburg, MD), Boehringer (Mannheim, FRG). Trypsin and lima bean trypsin inhibitor were purchased from Worthington (Freehold, NJ) and Stratagene, respectively. Grace's Antheraea medium (Grace, 1962) was purchased from Gibco (Grand Island, NY). Yeastolate and lactalbumin hydrolysate were from Difco Laboratories (Detroit, MI), and Fungibact antibiotic mix (100 000 units/mL penicillin, 10 mg/mL streptomycin, and 25 μg/mL fungizone) was from Irvine Scientific (Santa Ana, CA). Oligonucleotides were synthesized at the Howard Hughes Biopolymer Synthesis Facility at the University of Washington, Seattle, WA. *Sf9* cells and plasmid pVL 941 were generous gifts from Dr. Max Summers, Texas A&M University; the EGFR kinase domain was a gift of Dr. M. Mohammadi, New York University.

Construction of Plasmids. Open reading frames of TC.PTPase and TCΔC11.PTPase (Cool et al., 1990) were isolated by a *Tha*I/*Ssp*I digest and cloned into the unique *Bam*HI site of the plasmid pVL 941 (Luckow & Summers, 1989) after filling in the sticky ends with Klenow fragment and deoxynucleotides (Pharmacia). Correct orientation of insertion was confirmed by DNA sequence analysis (Sanger et al., 1977).

Cell Culture and Virus Purification. Sf9 cells were maintained in monolayer cultures as described (Summers & Smith, 1987). Cells were grown in Grace's Antheraea medium (Grace, 1962) supplemented with 3.3 g/L yeastolate, 3.3 g/L lactalbumin hydrolysate, 10% fetal calf serum (Hink, 1970), 100 units/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL fungizone. Cells were cotransfected with 1 μg of Ac-NPV DNA and 2 μg of recombinant plasmid as described (Summers & Smith, 1987). Purification of recombinant viruses was achieved by five rounds of serial end-point dilution and dot hybridization using ^{32}P-labeled TC.PTPase cDNA as a probe. The purity of the final virus suspension was checked by hybridization with a ^{32}P-labeled oligonucleotide probe; its sequence consists of nucleotides 37–66 of the Ac-NPV polyhedrin gene (Iddekinge et al., 1983), a portion missing in the pVL 941 expression vector. Extracts of *Sf9* cells infected with pure recombinant virus do not hybridize with this oligonucleotide.

Extraction of Sf9 Cells and Western Blot Analysis. Cells were infected at a high (>3) multiplicity of infection and grown at 27 °C. At different times postinfection, cells were harvested by centrifugation for 5 min at 5000*g*. The following buffers were routinely used for extraction: (a) low-salt buffer consisting of 25 mM imidazole, pH 7.2, 2 mM EDTA, 0.1% 2-mercaptoethanol, 1 mM benzamidine, 0.002% phenylmethanesulfonyl fluoride, 2 μg/mL leupeptin, 1 μg/mL pepstatin, 5 kallikrein units/mL aprotinin; (b) buffer containing additionally 0.5% Triton X-100; (c) buffer containing additionally 0.6 M KCl and 0.5% Triton X-100 or 1% CHAPS.

Cells were suspended in low-salt buffer (3×10^7 cells/mL) and disrupted by 30 strokes in a Dounce homogenizer on ice. After 10-min centrifugation at 10000*g*, the pellet was resuspended in half of the original volume of low-salt buffer containing 0.5% Triton X-100. The suspension was homogenized as previously described and centrifuged for 10 min at 100000*g*. Finally, the pellet was resuspended in high-salt buffer containing 0.5% Triton X-100 or 1% CHAPS. The homogenate was again centrifuged for 10 min at 100000*g*; the supernatants were used for Western blot analysis, protein purification, and PTPase assays. For Western blot analysis, proteins were subjected to SDS–PAGE as described (Laemmli, 1970) and electrophoretically transferred to nitrocellulose. Rabbit antibody 8172 raised against a synthetic peptide derived from the amino-terminal region of PTPase 1B (Cool et al., 1990) was used for detection in Western blots. Goat anti-rabbit IgG conjugated to alkaline phosphatase (Bio-Rad Laboratories, Richmond, CA) was used as a secondary antibody according to the manufacturer's instructions.

For purification of TC.PTPase, the Triton/KCl extract was made 20% in ammonium sulfate and centrifuged for 10 min at 10000*g*. The precipitate was resuspended in 200 μL of high-salt buffer containing 0.5% Triton X-100 and applied to a Superose 12 FPLC column equilibrated in the same buffer. For purification of the C-terminal truncated enzyme, the low-salt buffer extract was directly applied to a Sephadex G75 Superfine column (2.6 cm × 82.5 cm, flow rate ca. 10 mL/h). Peak fractions were made 20% in glycerol and frozen at −70 °C.

PTPase Assays. PTPase activity was measured as described (Tonks et al., 1988a), using saturating concentrations of tyrosyl-phosphorylated RCML and MBP as substrates. Trypsin treatment was carried out by incubating the PTPase (<0.25 unit/mL) in 40 μL of assay buffer with 1 μg of trypsin for 5 min at 30 °C. Trypsin was inhibited by addition of 6 μg of lima bean trypsin inhibitor, and the phosphatase reaction was started by addition of substrate. One unit of PTPase activity is defined as the amount of enzyme that releases 1 nmol of phosphate per minute. Protein concentration was determined with bovine serum albumin as the standard (Bradford, 1976).

Dephosphorylation of EGFR. The internal kinase domain of the EGFR as expressed in the baculovirus system (Hsu et al., 1990) was autophosphorylated in 20 mM HEPES, pH 7.5, 0.1% 2-mercaptoethanol, 5% glycerol, 1 μg/mL pepstatin, 5 kallikrein units/mL aprotinin, 2 μg/mL leupeptin, 5 mM manganese acetate, and 0.1 mM ATP (2.2×10^5 cpm/pmol) for 10 min at 30 °C, resulting in the incorporation of 0.7

[1] Ac-NPV, *Autographa californica* nuclear polyhedrosis virus; BHK cells, baby hamster kidney cells; BV, baculovirus; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; EGFR, epidermal growth factor receptor; FPLC, fast performance liquid chromatography; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; MBP, myelin basic protein; RCML, reduced, carboxamidomethylated, and maleylated lysozyme; *Sf9* cells, *Spodoptera frugiperda* cells; TC.PTPase, T-cell protein-tyrosine-phosphatase; TCΔC11.PTPase, C-terminal truncated form of TC.PTPase.

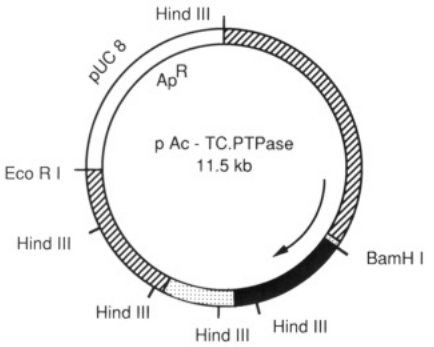


FIGURE 1: Restriction map of plasmid pAc-TC.PTPase. Open region, pUC 8 DNA; dotted regions, Ac-NPV polyhedrin promoter and structural gene; black region, TC.PTPase cDNA or TCΔC11.PTPase cDNA; hatched regions, genomic Ac-NPV DNA flanking the polyhedrin gene. The arrow shows the direction of transcription from the polyhedrin promoter.

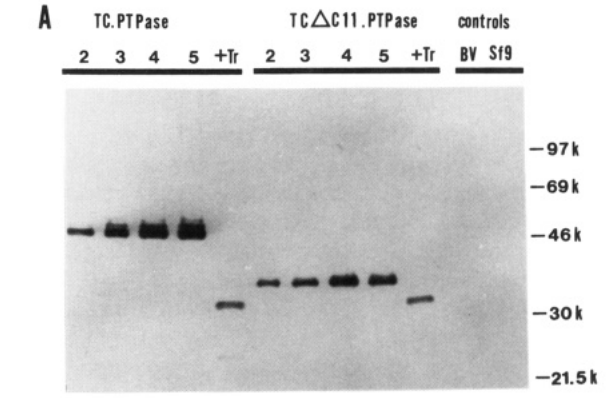
mol/mol of phosphate. The kinase reaction was stopped by adding EDTA to a concentration of 10 mM. The autophosphorylated receptor (80 ng) was then incubated either with buffer or with 100 ng of TC.PTPase or TCΔC11.PTPase, respectively, for 20 min at 30 °C. Sample buffer was added and the reaction run onto a 7.5% SDS–PAGE. The gel was dried and autoradiographed.

Results

Time Course of PTPase Expression. A *Tha*I/*Ssp*I fragment containing the open reading frame of the TC.PTPase cDNA (Cool et al., 1989) was cloned into the *Bam*HI site of the plasmid pVL 941 (Luckow & Summers, 1989). *Sf9* cells were cotransfected with pAc-TC.PTPase plasmid DNA (Figure 1) and Ac-NPV wild-type DNA. At different times postinfection, cells were harvested and lysed; the lysates were subjected to SDS–PAGE and immunoblot analysis. Uninfected cells or cells infected with wild-type (wt) Ac-NPV served as controls. Figure 2A shows the expression time course for TC.PTPase and TCΔC11.PTPase. The expression level of both forms increased steadily from day 2 to 5; after 5 days, the majority of cells had lysed due to the viral infection. Additional bands of higher molecular weight appeared after 3 days, probably due to some posttranslational modification. Limited trypsinolysis of both proteins gave rise to a tryptic fragment of approximately 33 kDa. Since antibody 8172 recognizes a sequence near the N-terminus of the enzyme, the main tryptic cleavage must have occurred in the C-terminal region of the protein. No cross-reacting material was detected in uninfected cells or in cells infected with the wt virus. Figure 2B illustrates the time dependence of PTPase activity in extracts from infected *Sf9* cells. The increase in activity paralleled the increased level of expression as observed by Western blot analysis. Substantial activity of the TC.PTPase toward RCML could be seen only following limited trypsinolysis of the enzyme.

Extraction of the Enzymes. The cells were extracted first in low-salt buffer, and the suspension was centrifuged. The pellet was reextracted with buffer containing 0.5% Triton X-100; this suspension was recentrifuged, and the second pellet was extracted with the same Triton buffer but in the presence of 0.6 M KCl. The full-length enzyme could be solubilized only by such a combination of salt and detergent (Figure 3); in contrast, the truncated form was readily soluble in aqueous buffer.

Purification of the Full-Length TC.PTPase. The TC.PTPase from Triton/KCl extracts was precipitated by adding ammonium sulfate to 20% saturation. The suspension was



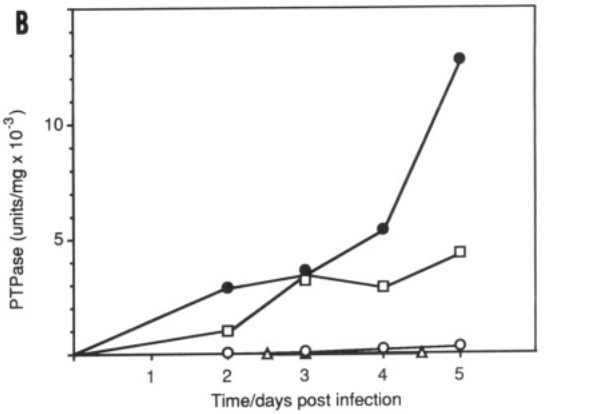


FIGURE 2: (A) Western blot of *Sf9* extracts. Protein (0.5 μg) was subjected to SDS–PAGE, blotted onto nitrocellulose, and probed with anti-PTPase peptide antibody 8172. Cells are expressing the full-length TC.PTPase (extracted in CHAPS/KCl buffer) or the truncated TCΔC11.PTPase (extracted in low salt buffer). BV, cells infected with wt baculovirus; Sf9, uninfected cells (both extracted in CHAPS/KCl buffer). Lanes 2–5, extracts from cells harvested 2-, 3-, 4-, 5-days postinfection; +Tr, extracts 3-days postinfection subjected to limited trypsinolysis. (B) Specific activity of PTPase toward RCML in *Sf9* cell extracts. Cells expressing (○) TC.PTPase (CHAPS/KCl buffer), (●) TC.PTPase after limited trypsinolysis, (□) TCΔC11.PTPase (low-salt buffer), and (△) control cells infected with wt baculovirus (CHAPS/KCl buffer).

centrifuged and the pellet solubilized in Triton/KCl buffer. Although the recovery was only ca. 50%, this step was necessary to concentrate the enzyme. The protein solution was applied to a Superose 12 FPLC column. TC.PTPase eluted as one major peak of activity with a trailing shoulder (Figure 4A). When fractions from both (18–20 and 21–24) were collected, the material in each pool resulted in the same band pattern on SDS–PAGE displaying a doublet at 48 kDa and a faint band at 50 kDa. Due to contaminants, the specific activity of the material in the trailing edge was approximately half that of the forward peak. Both fractions eluted at molecular weights (220K and 160K, respectively) higher than expected for the monomeric molecule (48K), implying aggregation, insertion into detergent micelles, or asymmetry of the molecule.

Aggregation of the protein could not be demonstrated directly. Assuming that the activation of the enzyme by polyamines or MBP (see below and Table III) resulted from disaggregation the full-length PTPase was chromatographed on the same FPLC Superose 12 column after equilibration of the column in 2 mM spermine or after preincubation with a 10-fold molar excess of unphosphorylated MBP. No shift in the elution pattern was observed. Furthermore, attempts at cross-linking with dimethyl suberimidate revealed no protein

Table I: Purification of Expressed TC.PTPase and TCΔC11.PTPase

	volume (mL)	total act. (units)	protein (mg)	sp. act. (units/mg)	purification (*n*-fold)	yield (%)
			(A) TC.PTPase[a]			
Triton/KCl extract	1.0	12100	4.6	2650	1	100
$(NH_4)_2SO_4$ precipitate	0.2	6400	2.3	2850	1	53
Superose 12 peak	0.5	3250	0.3	10700	4	27
			(B) TCΔC11.PTPase[b]			
extract	3.2	14900	4.65	3200	1	100
G75 Sephadex peak	9.0	7200	0.24	30200	9	48

[a] Activities determined with MBP as substrate in the presence of 5 mM EDTA. [b] Activities determined with RCML as substrate in the presence of 5 mM EDTA.

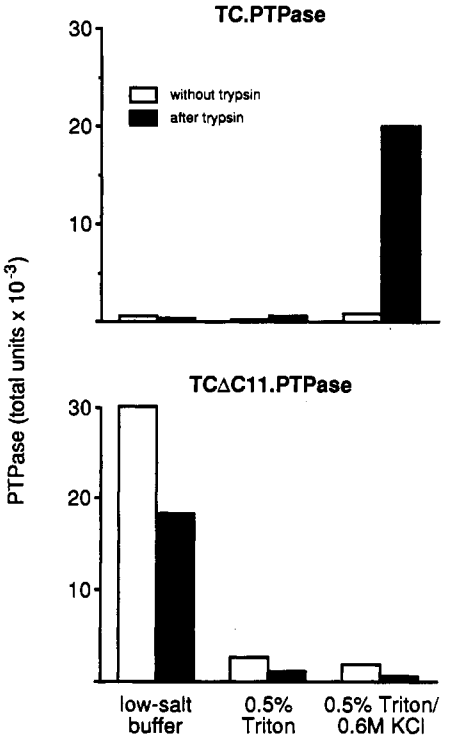


FIGURE 3: Distribution of full-length and truncated PTPases. Cells (6×10^7) were sequentially extracted as described in the text. PTPase activity was measured with RCML.

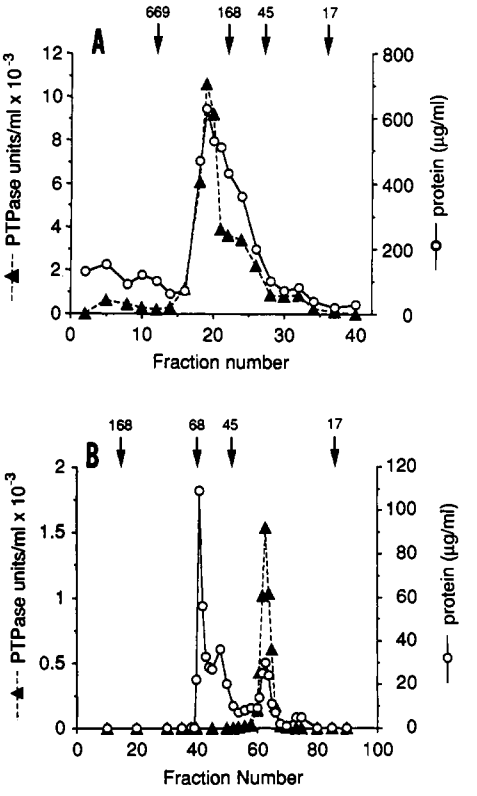


FIGURE 4: Purification of full-length and truncated PTPases. (A) Gel filtration on Superose 12 of TC.PTPase extract (in Triton/KCl buffer) after ammonium sulfate precipitation. Activities were determined with MBP as the substrate in the presence of 5 mM EDTA. (B) Gel filtration on Sephadex G75 Superfine of TCΔC11.PTPase extract (low-salt buffer). Activities were measured with RCML in the presence of 5 mM EDTA. Molecular weights of markers are indicated by arrows: thyroglobulin (669K) γ-globulin (168K), ovalbumin (45K), and myoglobin (17K).

species with a molecular weight higher than 48K on SDS–PAGE, whereas cross-linking of hemoglobin as a control under the same conditions was successful (data not shown).

To test the possible influence of the detergent, chromatography was also carried out in CHAPS, which forms smaller micelles (ca. 5 kDa) than Triton X-100 (ca. 90 kDa). However, here again, the enzyme eluted at a molecular weight higher than expected (ca. 170K).

While no explanation for the observed elution of the TC. PTPase at high molecular weight is supported by direct evidence, it is assumed that it is due to an asymmetry of the molecule. This assumption will not be easily confirmed since the enzyme is only soluble in the presence of detergent.

All three bands visible in SDS–PAGE correspond to different forms of the TC.PTPase since they are recognized by antibody 8172 (Figure 2A) and two other antibodies directed against different segments of the T-cell PTPase (residues 342–357 and 369–381; data not shown). Further characterization of the enzyme described below was performed on material obtained from peak fractions 19 and 20.

Purification of the Truncated Enzyme. Since 90% of TCΔC11.PTPase distributed in the aqueous buffer, the extract could be applied directly to a Sephadex G75 Superfine column (Figure 4B). A minor, trailing band (5–10%) can be seen in Figure 5. In contrast to the full-length protein, TCΔC11.PTPase eluted at its expected molecular weight. Table I summarizes the purification of both forms of the T-cell PTPase. These could be stored for months in the presence of 20% glycerol at −70 °C without significant loss of activity.

Substrate Specificity of PTPase Forms. Both forms of the T-cell PTPase were totally specific for phosphotyrosyl residues showing no activity toward MBP or histones phosphorylated by the cAMP-dependent protein kinase. The truncated form of the PTPase displayed a specific activity of 26000 units/mg toward tyrosyl-phosphorylated RCML. By contrast, the full-length enzyme was far less active (850 units/mg), suggesting that enzyme activity is repressed by the C-terminal segment (Table II). Both limited trypsinolysis and truncation of the molecule by introduction of a premature stop codon led to a 30-fold increase in activity toward RCML.

The activity of the full-length enzyme depended greatly on the nature of the substrate. In the presence of phosphorylated

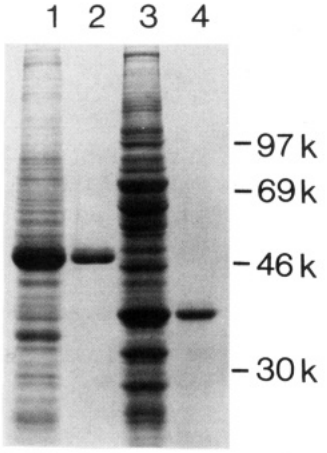


FIGURE 5: SDS–PAGE of cell extracts and purified enzymes. Lane 1, 10 μg of Triton/KCl extract of *Sf9* cells expressing TC.PTPase; lane 2; 1 μg of purified TC.PTPase; lane 3, 20 μg of low-salt buffer extract of cells expressing TCΔC11.PTPase; lane 4, 1 μg of purified TCΔC11.PTPase. Molecular weights of markers are shown on the right. The gels in lanes 2 and 4 were overloaded to reveal possible contaminants; under these conditions, the doublet at 48 kDa of the full-length enzyme (lane 2) is not resolved.

Table II: Enzymatic Properties of PTPase Forms

	RCML		MBP	
	sp. act.[a] (units/mg)	K_m (nM)	sp. act.[a] (units/mg)	K_m (nM)
TC.PTPase	850 ± 170	200	10300 ± 1300	500
after trypsinolysis	23300 ± 3800	nd[b]	3600 ± 1000	nd
TCΔC11.PTPase	26000 ± 3000	50	4700 ± 500	1250
after trypsinolysis	15300 ± 2600	nd	1900 ± 200	nd

[a] Average and standard deviations for three separate preparations measured under conditions of substrate saturation in the presence of 5 mM EDTA. [b] Not determined.

MBP, it displayed a specific activity of 10 300 units/mg as compared to 4700 units/mg only for the truncated form. These data suggest that MBP interacts with the C-terminal segment, resulting in an activation of the enzyme. Both forms of the T-cell PTPase readily dephosphorylated the soluble kinase domain of the EGFR following its autophosphorylation (Figure 6).

Influence of Various Effectors on PTPase Activity. As with placenta PTPase 1B (Tonks et al., 1988b), both forms of the T-cell enzyme were inhibited by micromolar concentrations

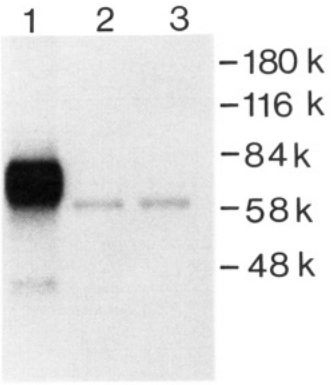


FIGURE 6: Dephosphorylation of the cytoplasmic domain of the EGFR. Eighty nanograms of the kinase domain of the EGFR following autophosphorylation was incubated with (lane 1) buffer, (lane 2) 100 ng of TC.PTPase, and (lane 3) 100 ng of TCΔC11.PTPase. Molecular weights of markers are indicated on the right.

of the classical inhibitors vanadate (Swarup et al., 1982), molybdate, and Zn^{2+} (Brautigan et al., 1981) (Table III). Calcium and magnesium were essentially without effect. Both forms of the T-cell enzyme were inhibited by nanomolar concentrations of the polyanions; this effect was only observed with RCML as substrate. The enzyme was activated by EDTA but not as potently as PTPase 1B (Tonks et al., 1988b). The full-length enzyme was markedly activated by polycationic compounds (up to 7-fold by spermine and up to 3-fold by unphosphorylated MBP) only when negatively charged RCML was used as the substrate. By contrast, activation of the truncated form of the enzyme by these compounds was 30% at most, suggesting that the polycationic molecules interact with the C-terminal region.

Discussion

In this paper, we report the first characterization of a member of the group of low molecular weight (ca. 50K) PTPases. Two forms of the T-cell PTPase were purified from baculovirus-infected *Sf9* cells. Up to 100 μg of enzyme could be purified from a single tissue culture flask containing 6×10^7 cells. The cells were harvested after 72 h to minimize proteolysis and the accumulation of posttranslational modification products of the expressed proteins. Proteolytic fragments increased with time and partitioned in the aqueous buffer in contrast to the full-length enzyme, while higher molecular weight bands began to appear 3 days postinfection.

Table III: Effectors of PTPase Activity[a]

enzyme form:	TC.PTPase		TCΔC11.PTPase	
substrate:	RCML	MBP	RCML	MBP
effector				
none	100	100	100	100
100 μM vanadate	0	0	2	1
10 μM molybdate	0	1	2	2
100 μM Zn^{2+}	33	101	15	66
0.01 μM heparin	96	120	58	102
1 μM heparin	8	108	8	75
10 μM heparin	5	73	1	90
0.01 μM poly(Glu/Tyr) 4:1	66	119	70	102
1 μM poly(Glu/Tyr) 4:1	18	133	17	104
10 μM poly(Glu/Tyr) 4:1	6	58	11	127
5 mM EDTA	124	137	170	150
2 mM spermine	727	62	110	97
2 mM spermidine	249	43	132	108
1 μM unphosphorylated MBP	122	62	110	97
10 μM unphosphorylated MBP	297	43	132	108
50 μM unphosphorylated MBP	279	15	10	66

[a] Activity is expressed as the percentage of phosphate released relative to control in which the effector was omitted. All assays were performed in duplicate in the presence of 5 μM substrate.

In SDS–PAGE, purified fractions of the full-length T-cell PTPase showed a doublet (not resolved in Figure 5) at 48 kDa and a faint band at 50 kDa. All three bands cross-react immunologically.

The nature of this molecular heterogeneity is not known. It was not due to phosphorylation since infection of the *Sf9* cells in the presence of [^{32}P]P_i did not result in phosphate incorporation. Furthermore, treatment of the purified PTPase with the serine/threonine type 1 and 2A protein phosphatases, as well as potato acid phosphatase, did not alter the band pattern. Infection of *Sf9* cells in the presence of 1 μg/mL tunicamycin (which blocks N-linked glycosylation; Duksin & Mahoney, 1981) was without effect. Finally, fatty acylation as observed in a number of proteins [see review by Schultz et al. (1988)] seems unlikely since the T-cell PTPase does not fulfill the structural requirements for either myristoylation (an N-terminal glycine; Schultz et al., 1988) or isoprenylation (a C-terminal CAAX box; Glomset et al., 1990).

The 37-kDa truncated form of the PTPase also showed some degree of molecular heterogeneity, namely, a minor, trailing band recognized by peptide antibody 8172. The intensity of this band appears to increase upon storage.

As shown, the full-length PTPase prefers MBP over RCML as a substrate, in contrast to the C-terminal truncated form which favors RCML over MBP. This suggests an interaction with MBP at a site within the C-terminal segment. Such an interaction might explain the activation of the full-length T-cell PTPase by unphosphorylated MBP. Curiously, however, purified placenta PTPase 1B, also truncated at the C-terminus, displays almost the same activity toward both substrates and even shows some activation by unphosphorylated MBP (Tonks et al., 1988b).

While the kinetic properties of the T-cell PTPase were studied with two artificial substrates, a physiological substrate for this enzyme had not been identified thus far. The kinase domain of the EGFR includes five major autophosphorylation sites (Margolis et al., 1989). The autophosphorylated cytoplasmic domain of the EGFR did serve as a substrate for both forms of the T-cell PTPase. Under the conditions of the experiment (high enzyme/substrate ratio), dephosphorylation was essentially quantitative. Due to limited availability of this substrate, no detailed kinetic data are available as yet.

When subjected to limited trypsinolysis, the full-length and truncated T-cell PTPases generate similar fragments of ca. 33 kDa (Figure 2A). However, the specific activity of the material obtained from the truncated enzyme is somewhat lower than that generated from the full-length protein. A possible explanation is that the truncated enzyme is more susceptible to proteolytic cleavage, thus generating inactive fragments not recognized by the peptide antibody on Western analysis.

The C-terminal region of the TC.PTPase is responsible for localization of the enzyme in the particulate fraction of *Sf9* cells, as also observed with BHK cells (Cool et al., 1990). This complicates a comparison of the properties of the two purified forms of the enzyme because of the different environments in which they must be kept. While the truncated enzyme is soluble in aqueous buffers, the full-length enzyme requires detergent to remain in solution. Decreasing the detergent concentration below the critical micellar concentration leads to precipitation and almost total inactivation of the protein.

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Evidence for a "Cysteine-Histidine Box" Metal-Binding Site in an *Escherichia coli* Aminoacyl-tRNA Synthetase†

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ABSTRACT: *Escherichia coli* alanyl-tRNA synthetase contains the sequence Cys-X_2-Cys-X_6-His-X_2-His. This motif is distinct from the zinc fingers of DNA-binding proteins but has some similarity to the Cys-X_2-Cys-X_4-His-X_4-Cys zinc-binding motif of retroviral *gag* proteins, where it has a role in RNA packaging. In Ala-tRNA synthetase, this sequence is located in an amino-terminal domain which has the site for docking the acceptor end of the tRNA near the bound aminoacyl adenylate and is immediately adjacent in the sequence to the location of a mutation that affects the specificity of tRNA recognition. We show here that Ala-tRNA synthetase contains approximately 1 mol of zinc/mol of polypeptide and that addition of the zinc chelator 1,10-phenanthroline inhibits its aminoacylation activity. Conservative mutations of specific cysteine or histidine residues in the "Cys-His box" destabilize and inactivate the enzyme, whereas mutations of intervening amino acids do not inactivate. The possibility that this motif can bind zinc (or cobalt) was demonstrated with a synthetic 22 amino acid peptide that is based on the sequence of the alanine enzyme. The peptide–cobalt complex has the spectral characteristics of tetrahedral coordination geometry. The results establish that the Cys-His box motif of Ala-tRNA synthetase has the potential to form a specific complex with zinc (at least in the context of a synthetic peptide analogue) and suggest that this motif is important for enzyme stability/activity.

Although a wide variety of cellular and viral proteins interact with RNA, relatively few protein sequence motifs for RNA binding have been identified. An "RNP consensus" sequence has been implicated in RNA binding in many nuclear and cytoplasmic proteins which bind to mRNA, pre-mRNA, snRNA, and pre-RNA (Mattaj, 1989; Bandziulis et al., 1989). A crystal structure of the RNA-binding domain of U1 snRNP A shows that these RNP consensus sequences are contained within a four-stranded β-sheet at the presumed RNA-binding site (Nagai et al., 1990). The *gag* proteins of retroviruses are required for viral RNA packaging, and a three-dimensional structure of a member of this family has been solved (Summers et al., 1990). However, it is not known how these proteins interact with RNA. In contrast, several well-characterized DNA-binding motifs have been identified (e.g. the "leucine zipper," "helix–turn–helix", "helix–loop–helix", and "zinc finger"); three-dimensional structures of these motifs have been solved, and DNA recognition has been characterized at the molecular level in many cases (Struhl, 1989).

The most detailed information about protein–RNA recognition comes from the recently solved structure of the cocrystal of *Escherichia coli* glutaminyl-tRNA synthetase and $tRNA^{Gln}$ (Rould et al., 1989). Sequence-specific contacts with the acceptor stem of $tRNA^{Gln}$ are made by a domain that is inserted between the first and second halves of a dinucleotide (Rossmann) fold. This domain contains 110 amino acid residues and is composed of a five-stranded antiparallel β-sheet flanked by three α-helices. The structure of $tRNA^{Gln}$ is altered upon binding to this domain of Gln-tRNA synthetase; the terminal base pair is broken and the 3′-end of the tRNA is folded back on the rest of the structure.

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