Catalytic Domains of the LAR and CD45 Protein Tyrosine Phosphatases from *Escherichia coli* Expression Systems: Purification and Characterization for Specificity and Mechanism†

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ABSTRACT: The cytoplasmic domains of two human transmembrane protein tyrosine phosphatases (PTPases), LAR and CD45, have been expressed in *Escherichia coli*, purified to near-homogeneity, and compared for catalytic efficiency toward several phosphotyrosine-containing peptide substrates. A 615-residue LAR fragment (LAR-D1D2) containing both tandemly repeated PTPase domains shows almost identical specific activity and high catalytic efficiency as the 40-kDa single-domain LAR-D1 fragment, consistent with a single functional active site in the 70-kDa LAR-D1D2 enzyme. A 90-kDa fragment of the human leukocyte CD45 PTPase, containing two similar tandemly repeated PTPase domains, shows parallel specificity to LAR-D1 and LAR-D1D2 with a high k_{cat}/K_m value for a phosphotyrosyl undecapeptide. Sufficient purified LAR-D1 and LAR-D1D2 PTPases were available to demonstrate enzymatic exchange of ^{18}O from $^{18}O_4$ inorganic phosphate into $H_2{}^{16}O$ at rates of $\sim 1 \times 10^{-2}$ s^{-1}. The oxygen-18 exchange probably proceeds via a phosphoenzyme intermediate. Brief incubation of all three PTPase fragments with a [^{32}P]phosphotyrosyl peptide substrate prior to quench with SDS sample buffer and gel electrophoresis led to autoradiographic detection of ^{32}P-labeled enzymes. Pulse/chase studies on the LAR ^{32}P-enzyme showed turnover of the labeled phosphoryl group.

The enzyme-mediated transfer of phosphoryl groups to and from specific tyrosyl residues in particular subsets of proteins occurs in many intracellular signaling pathways in hormone and growth factor initiated events as well as in control of cell cycling signals (Alexander, 1990). The level of tyrosyl group phosphorylation is regulated by the balance of protein tyrosine kinases (PTKs)[1] and protein tyrosine phosphatases (PTPases). An imbalance in the favor of excess tyrosine kinase activity (e.g., members of the src kinase family) can lead to cell transformation (Cantley et al., 1991) while an excess of PTPase activity can reverse such a phenotype and suggests some PTPase genes function as anti-oncogenes. Two types of PTPases have so far been detected; one group is largely cytoplasmic although perhaps with a hydrophobic C-terminus (Brown-Shimer et al., 1990) while the second group are transmembrane proteins (Saito & Streuli, 1991). The transmembrane PTPases, with variable extracellular domains, a transmembrane domain, and cytoplasmic catalytic domains, have been termed receptor PTPases (Saito & Streuli, 1991) whose activity and/or specificity could be regulated by particular extracellular ligands. The prototypic receptor PTPase has been the 180–220-kDa CD45 cell-surface antigen, also known as leukocyte common antigen (LCA) because of its expression on cells of hematopoeitic origin (Charbonneau et al., 1988). LCA exists in at least five human isoforms arising by alternate splicing of three exons in the extracellular domain to a common catalytic domain (Streuli et al., 1987; Thomas, 1989). Another receptor PTPase more widely expressed in many cell types is the homologous LAR (leukocyte antigen related) PTPase (Streuli et al., 1988, 1989), and by now at least 10 human PTPases are known, doubtless with more to be identified (Krueger et al., 1990; Saito & Streuli, 1991).

While understanding of the role of individual receptor PTPases awaits identification of particular extracellular regulatory ligands, rather little is yet known about the catalytic domains or their specificity for specific phosphotyrosyl-containing sequences in potential protein substrates. We have recently purified to homogeneity from *Escherichia coli* expression system a 40-kDa soluble fragment of one of the two tandemly duplicated domains of the human LAR PTPase. This LAR-D1 construct was characterized for specificity and catalytic efficiency (Cho et al., 1991). To compare the one-domain 40-kDa catalytic fragment of LAR with both of the tandemly duplicated PTPase domains encoding all 607 amino acids of the cytoplasmic domain protein of LAR, we describe here the purification and characterization of a 70-kDa LAR-D1D2 fragment. For further evaluation of the specificity of catalytic domains of receptor PTPases, we also report the partial purification and evaluation of the 707-residue cyto-

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[1] Abbreviations: PTPase, protein tyrosine phosphatase; PTK, protein tyrosine kinase; SDS, sodium dodecyl sulfate; LCA, leukocyte common antigen; NMR, nuclear magnetic resonance; EDTA, ethylenediaminetetraacetic acid; β-ME, β-mercaptoethanol; DTT, dithiothreitol; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; EGFR, epidermal growth factor receptor; IR, insulin receptor; HPLC, high-performance liquid chromatography; pEGFR, TAENAEpYLRVA; IR-1, TRDlpYETDYYRK; IR-2, TRDIYETDpYYRK; IR-3, TRDIYETDYpYRK; lck394, EDNEpYTARE; lck505, TEGQpYQPQP; src527, TEPQpYQPGE.

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plasmic fragment of CD45 (LCA) PTPase and assessment of catalytic efficiency of all three forms of PTPases with specific phosphotyrosine peptide substrates. We also report initial mechanistic results consistent with phosphoenzyme intermediates.

Materials and Methods

General. Fmoc amino acids for peptide synthesis were from Pennisula Laboratories. Fmoc-tyrosine was from Bachem Bioscience. BOP and HOBT were from MilliGen Biosearch whereas HBTU and TPTU were prepared as previously described (Knorr et al., 1989). Bovine serum albumin was fraction V powder from Pentex Inc. All other chemicals and reagents were from Aldrich or Sigma. DNase was grade DP from Worthington/Cooper. Protein concentrations were determined using the Bio-Rad dye reagent (catalogue no. 500-0006) following the manufacturer's directions with bovine serum albumin as the standard. Affi-Gel Blue was purchased from Bio-Rad. DEAE-Sepharose and a prepacked phenyl-Superose column were from Pharmacia LKB Biotechnology. [γ-^{32}P]ATP was from Amersham or ICN Biochemicals. Dialysis tubing was from Spectrum. NMR spectra were recorded at 200 MHz at Harvard Medical School NMR center.

Buffers. Buffers used were as follows: (A) 33 mM Tris·HCl, 2 mM EDTA, and 10 mM β-ME, pH 8.0; (B) 33 mM imidazole hydrochloride, 2 mM EDTA, and 10 mM β-ME, pH 7.0; (C) 25 mM Hepes, 2 mM EDTA, 10 mM DTT, pH 7.3, and 1 mg/mL bovine serum albumin; (D) 100 mM Mes, 2 mM EDTA, and 10 mM DTT, pH 6.0.

Expression of the Active Catalytic Domains of PTPases (LAR-D1, LAR-D1D2, and CD45-D1D2) in E. coli. pT7-LAR-D1 plasmid that encodes the catalytic domain of the human LAR PTPase has been described (Cho et al., 1991; Tsai et al., 1991). Essentially the segment of the human LAR PTPase cDNA encoding amino acid positions 1275–1613 was modified so that this sequence is flanked by a *Bam*HI site at the 5′ end and a termination codon and a *Hin*III site at the 3′ end. The *Bam*HI-*Hin*III fragment thus generated was inserted between the *Bam*HI and *Hin*III sites within the polylinker sequence in the pT7-7 prokaryotic expression vector. Thus, the LAR-D1 PTPase coding sequence is fused to nine amino acids (MARIRARGS) derived from the pT7-7 vector at the N-terminus, and two amino acids (GV) at the C-terminus.

pT7-LAR-D1D2 plasmid the encodes both D1 and D2 cytoplasmic domains of the human LAR PTPase was constructed as follows. The segment of the human LAR cDNA encoding the amino acid positions 1275 through the C-terminal amino acid of the LAR protein (1881) and a part of the 3′-noncoding sequence was inserted into the pT7-7 plasmid vector. Thus, the LAR-D1D2 PTPase coding sequence also contains the same nine amino acids derived from the pT7-7 vector at the N-terminus, but the C-terminus is identical to the human LAR protein.

pT7-CD45-D1D2 plasmid that encodes both D1 and D2 cytoplasmic domains of the human CD45 PTPase was constructed using cDNA encoding amino acid positions 584–1281. The 3′-noncoding sequence was modified so that this sequence is flanked by a *Bam*HI site at the 5′ end and by an *Eco*RI site in the 3′-noncoding region. The *Bam*HI–*Eco*RI fragment thus generated was inserted between the *Bam*HI and *Eco*RI sites of the pT7-7 prokaryotic expression vector. Thus, the CD45-D1D2 PTPase coding sequence is fused to eight amino acids (MARIRARG) derived from the pT7-7 vector at the N-terminus, and the C-terminus is identical to the human CD45 protein.

Purification of LAR-D1 PTPase. Purification of LAR-D1 PTPase was accomplished as reported (Cho et al., 1991). Enzyme of ca. 90% purity was combined and concentrated (Amicon YM-30) to ~5 mg/mL protein. Glycerol was added to a final concentration of 30% before being stored frozen at −80 °C.

Purification of LAR-D1D2 PTPase. The procedures for LAR-D1 purification also applied for purification of LAR-D1D2 PTPase except for the following modifications: 0–40% ammonium sulfate fraction was collected, and the fractions eluted with a buffer containing 10–30 mM NaCl from DEAE-cellulose column were collected.

Purification of the Cytosolic Domain of CD45. The crude lysate obtained from cultures of *E. coli* BL21(DE3) carrying the pT7-7 cytosolic CD45 fragment containing plasmid was fractionated with ammonium sulfate, and the 35–60% fraction was dialyzed against buffer A. The dialyzed sample was diluted with buffer B and loaded on a DEAE-Sepharose column (2.2 × 40 cm). It was eluted with buffer B with a gradient to buffer B containing 1 M NaCl. The active fraction (as determined by the appearance of a yellow color upon addition to 100 mM *p*-nitrophenyl phosphate in buffer B), eluted with a buffer containing approximately 0.2 M NaCl, were collected and concentrated (Amicon YM-30). The resulting sample was dialyzed against buffer A and loaded on a Affi-Gel Blue column (1.6 × 17 cm). After loading was complete, the column was washed with 50 mL of buffer A and eluted with a gradient in the same buffer containing 1 N NaCl. The active fractions were collected, concentrated, and dialyzed against buffer B. Ammonium sulfate was added to the sample to a concentration of 1 M and loaded on a phenyl-Superose column (1 × 10 cm) which was then washed with buffer B containing 1 M ammonium sulfate. The active fractions were combined and concentrated to ~3 mg/mL of protein. Glycerol was added to a final concentration of 30% before being stored frozen at −80 °C.

Synthesis of Fmoc-Tyr(PO_3Me_2)-OH and of Phosphotyrosine-Containing Peptides. Syntheses of Fmoc-Tyr-(PO_3Me_2)-OH and of phosphotyrosine-containing peptides were as described previously (Cho et al., 1991).

Assay for Tyrosine Phosphatase Activity. To assay the dephosphorylation of phosphotyrosine-containing peptides by LAR or CD45 PTPases, the release of inorganic phosphate was determined using a malachite green assay (Lanzetta et al., 1979; Martin et al., 1985). For this assay, the enzyme was diluted with buffer C and added to the reaction mixture containing the phosphopeptide in buffer D. Typically the total reaction volume was 50 μL after the addition of 5 μL (20–300 ng) of diluted enzyme, and the reaction was incubated at 25 °C for 1–2 min. The assay was quenched with 900 μL of the malachite green reagent (Lanzetta et al., 1979), and after 10 min at room temperature, the absorbance at 660 nm was determined. The nanomoles of P_i released was calculated by comparison to a standard curve determined for inorganic phosphate. For purification of enzymes, *p*-nitrophenol phosphate was used as a substrate for assay.

^{18}O Exchange from $KH_2P^{18}O_4$ into H_2O. $KH_2P^{18}O_4$ (77% isotopic purity) was prepared as described previously (Risley & Van Etten, 1978). Enzyme reaction mixture (51 mM $KH_2P^{18}O_4$ with 77% ^{18}O, 60 μL of 10 × buffer D, 17% D_2O, 0.5 mg of LAR-D1 or 1.1 mg of LAR-D1D2, and H_2O to 0.6 mL) was incubated at room temperature (25 °C), and ^{31}P NMR spectra were obtained. Peaks were integrated, and the values obtained were used for calculation. Control experiments were also carried out with the same mixture without enzyme.

The ^{18}O exchange rate was calculated according to the method of D. H. Eargle et al. (Eargle et al., 1977).

Preparation of $^{32}PO_4$-Tyrosyl Peptide. EGFR-11-mer peptide (TAENAEYLRVA) was labeled with ^{32}P on the tyrosine residue by the $p56^{lck}$ tyrosine kinase as follows. To 150 μL (125 μCi) of [γ-^{32}P]ATP (1250 Ci/mmol) was added 20 μL of 3 × kinase buffer (150 mM Hepes and 30 mM $MgCl_2$, pH 7.5) followed by 10 μL of $p56^{lck}$ (0.05 μg, specific activity 0.8 μmol min^{-1} mg^{-1}) (Ramer et al., 1991) and 10 μL of EGFR-11-mer peptide (10 mg/mL). Another 5 μL of $p56^{lck}$ was added 2 and 4 h later. After 18 h at room temperature, the reaction was quenched with 75 μL of 1 M KH_2PO_4 (pH 3.0), and the resulting mixture was purified (three 50-μL injections) by reverse-phase HPLC chromatography on a 3 mm × 3 cm Perkin-Elmer Pecosphere 3-Å C_{18} column with a 5-min linear gradient from 0.01% TFA in water to 0.01% TFA in 50% CH_3CN/H_2O at a flow rate of 3 mL/min. After removal of solvent, the $^{32}PO_4$-peptides were dissolved in H_2O. This gave 7 μCi of peptide with <1% contaminating [γ-^{32}P]ATP as determined by HPLC. The peptide obtained is a mixture of $^{32}PO_4$-peptide and unlabeled peptide.

Detection of Phosphoenzyme Intermediate. The reaction was initiated by addition of 1 μL of LAR-D1 (1 μg/μL) to a mixture of 4 μL of $^{32}PO_4$-EGFR peptide (1.1 μCi, 500 Ci/mmol), 3 μL of $^{31}PO_4$-EGFR peptide (83 μM, final specific activity 4.4 Ci/mmol), and 1 μL of 5 × PTPase buffer (0.5 M Mes, 25 mM EDTA, and 50 mM DTT, pH 6.0) preequilibrated at 4 °C. The reaction was quenched 10 s later by addition of 20 μL of 2 × SDS sample buffer. A control experiment was carried out by heating LAR-D1 for 5 min at 95 °C before addition to the reaction mixture. As a chase experiment, 10 μL of $^{31}PO_4$-EGFR peptide (83 μM) was added 20 s after the reaction was initiated, and the reaction was quenched 20 min later by addition of 20 μL of 2 × SDS sample buffer. The quenched mixtures were loaded on a 10% polyacrylamide gel, and electrophoresis was carried out at 4 °C. The gel was washed with deionized water for 15 min and dried, and ^{32}P-protein species were detected by autoradiography. After the autoradiogram was obtained, the part of the gel corresponding to the band was cut and counted by scintillation counter.

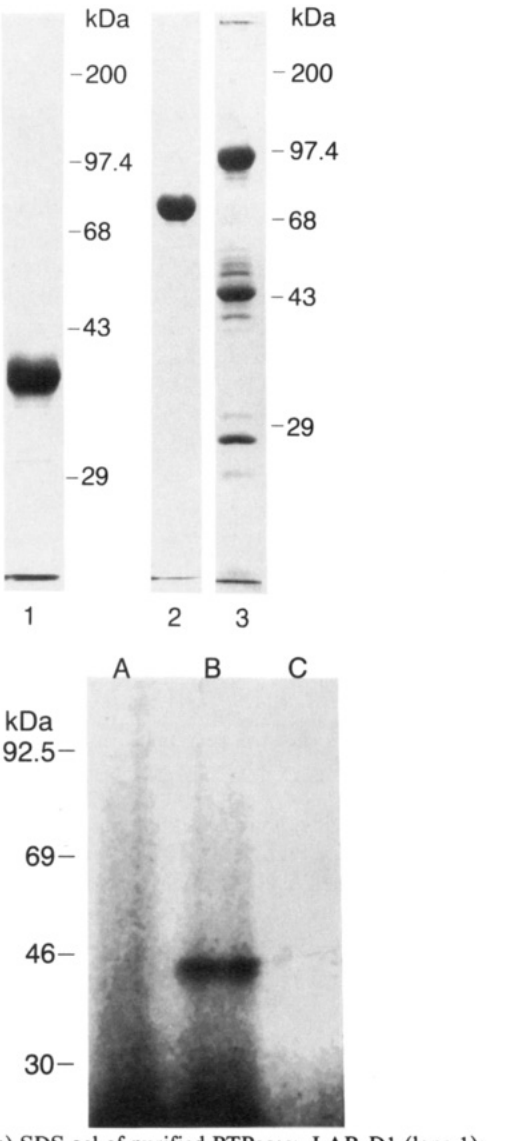


FIGURE 1: (Top) SDS gel of purified PTPases: LAR-D1 (lane 1); LAR-D1D2 (lane 2); and CD45-D1D2 (lane 3). LAR-D1 was electrophoresed on a 8% polyacrylamide gel and others on a 10% gel. (Bottom) Autoradiogram of ^{32}P-labeled LAR-D1: A, controls; B, ^{32}P-labeled LAR-D1; C, chase experiment (see Materials and Methods).

Results

Purification of the LAR-D1D2 Protein and Enzymatic Activity. The LAR-D1D2 domain comprising 607 amino acid residues [1275–1881 of full-length LAR (Streuli et al., 1988)] was expressed in *E. coli* on plasmid pT7 at a level of 8 mg/L. The LAR-D1D2 was purified to apparent homogeneity by variation of the method used by us to purify LAR-D1 (Cho et al., 1991) (Figure 1, top panel). The role of the tandemly repeated second domain has been unclear in the receptor PTPases. Mutational analysis by Streuli et al. (1990) had suggested PTPase domain D1 was responsible for the observed PTPase catalytic activity measured in crude extracts of *E. coli* with [^{32}P]phospho-Raytide or ^{32}P-phosphorylated myelin basic protein as substrate, but the possibility remained that the second domain (D2) modulated turnover rates or specificity. The purified LAR-D1D2 was assayed with five phosphotyrosyl peptide substrates (Cho et al., 1991), comprising the known autophosphorylation sites of EGF receptor, one of the phosphorylation sites of the insulin receptor, and the C-terminal autoregulatory phosphotyrosyl sites of the $p60^{src}$ and the $p56^{lck}$ members of the src tyrosine kinase family. The values k_{cat}, K_m, and k_{cat}/K_m are collected in Table I. The V_{max} values for LAR-D1D2 fall in a very narrow range of 22–36 μmol min^{-1} mg^{-1} corresponding to turnover numbers of 1500–2500 catalytic dephosphorylations/min, assuming one active site per LAR-D1D2 fragment. The K_m values vary ~30-fold from 27 μM for the IR-1 phosphotyrosyl peptide to 780 μM for the lck505 phosphotyrosyl peptide, and these ranges of K_m dominate the observed variations in the catalytic efficiency ratio k_{cat}/K_m, with a maximal value of 1.4×10^6 M^{-1} s^{-1}. A side by side comparison of LAR-D1D2 with LAR-D1 reveals the same rank order for the four peptides and quite comparable K_m values in particular. These results indicate that at the level of primary phosphotyrosine sequence recognition, the D2 domain of LAR plays no obvious role in modulation of specificity or turnover rates and suggest studies on the smaller LAR-D1 catalytic fragment may suffice for activity studies although specific full-length phosphotyrosyl protein substrates are yet to be compared with LAR-D1 vs LAR-D1D2.

Purification and Catalytic Activity of CD45-D1D2. The purification of the CD45 (LCA) 707-residue 2-domain catalytic fragment from the *E. coli* expression system is more difficult than either LAR fragment, and a 3-column purifi-

Table I: Substrate Specificity Data for Seven Peptides for LAR-D1, LAR-D1D2, and CD45-D1D2[a]

		LAR-D1	LAR-D1D2	CD45-D1D2
P-EGFR (TAENAEpYLRVA)	V_{max}	68	30	136
	K_m	0.48	0.42	0.16
	k_{cat}/K_m	8.5	8.6	128
IR-1 (TRDIpYET-DYYRK)	V_{max}	96	31	72
	K_m	0.027	0.027	0.034
	k_{cat}/K_m	240	140	318
IR-2 (TRDIYETD-pYYRK)	V_{max}	33		
	K_m	0.28		
	k_{cat}/K_m	7.8		
IR-3 (TRDIYETDY-pYRK)	V_{max}	45		134
	K_m	0.71		0.17
	k_{cat}/K_m	4.2		124
lck394 (EDNEpYTARE)	V_{max}	75		
	K_m	0.17		
	k_{cat}/K_m	29		
lck505 (TEGQpYQPQP)	V_{max}	27	36	66
	K_m	0.73	0.78	0.13
	k_{cat}/K_m	2.5	5.5	76
src527 (TEPQpYQPGE)	V_{max}	58	22	186
	K_m	0.35	0.17	0.16
	k_{cat}/K_m	11	15	174

[a] Units: V_{max}, micromoles per minute per milligram; K_m, millimolar; k_{cat}/K_m, $\times 10^4$ M^{-1} s^{-1}.

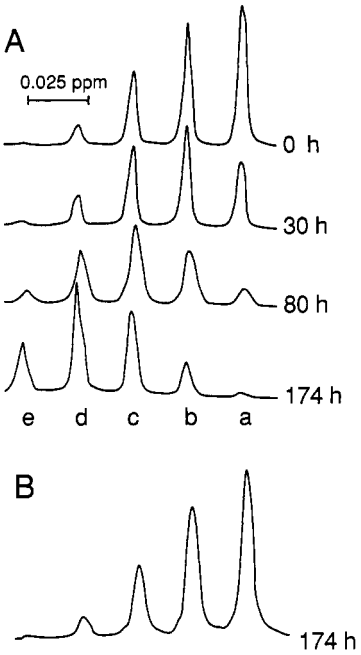


FIGURE 2: ^{31}P NMR spectra of ^{18}O exchange experiment with LAR-D1D2. (A) Spectra recorded at 0, 30, 80, and 174 h after the enzyme was added to the reaction mixture containing $P^{18}O_4$; (B) control experiment without enzyme recorded after 174 h. a, $P^{18}O_4$; b, $P^{18}O_3{}^{16}O$; c, $P^{18}O_2{}^{16}O_2$; d, $P^{18}O^{16}O_3$; e, $P^{18}O^{16}O_4$.

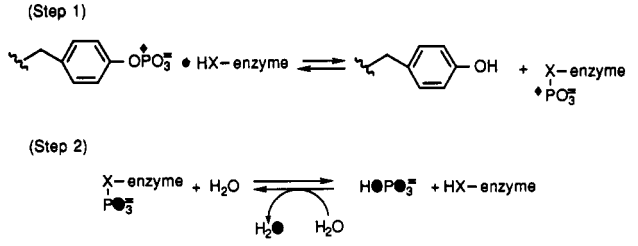
cation yields enzyme of 70% purity as estimated by SDS gel analysis (lane 3, Figure 1). The N-terminal sequence of the 90-kDa band was AXIRARGSXNLDEQXELV, as predicted from the gene sequence starting at the expected residue of CD45. CD45 of this purity served to assess catalytic specificity and efficiency against the common phosphotyrosyl peptides of Table I and for comparison with the LAR PTPase catalytic fragments. The V_{max} values for CD45 at 66–186 μmol min^{-1} mg^{-1} are about 2–9-fold higher than for LAR-D1D2. The K_m values of IR-1 pY peptide at 34 μM for CD45 are very close to the 27 μM LAR-D1D2 value. The other four pY peptides are all at 130–170 μM, not significantly different from the LAR K_m's with the possible exception of a 6-fold better recognition by CD45 of the lck505 pY peptide, a possibly relevant biological substrate. In general, the LAR and CD45 PTPases are about equivalent with these phosphotyrosyl peptides, but CD45 is some 2–15-fold more efficient by k_{cat}/K_m criteria.

Mechanistic Analysis of PTPase Action: Search for a Phosphoenzyme Intermediate. Both alkaline phosphatases and acid phosphatases, which have been well analyzed mechanistically, proceed via covalent phosphoenzyme intermediates (Schwartz et al., 1963; Van Etten & Hickey, 1977). The amino acid residue bound to the phosphoryl group in Enz–X–PO_3^- is serine in alkaline phosphatases (Schwartz et al., 1963) while in low molecular weight acid phosphatases histidine has been implicated (Van Etten & Hickey, 1977). Recently, Van Etten and colleagues (Zhang & VanEtten, 1991) have published a thorough analysis of a low molecular weight PTPase from beef heart in which compelling kinetic evidence for a phosphoenzyme intermediate was presented, including burst kinetics, phosphotransfer to alcohols as alternate acceptor substrates, and oxygen exchange from P_i to H_2O (Schwartz et al., 1963). We have examined the catalytic mechanism of purified LAR-D1 and D1D2. LAR-D1 has high turnover numbers (15–45 s^{-1}) with pY peptide substrates and also dephosphorylates *p*-nitrophenyl phosphate (PNP), with K_m = 1.73 mM and k_{cat} = 4.1 s^{-1} at pH 6.0, 25 °C. While we have not yet assessed for burst behavior by pre-steady-state kinetics, the LAR-D1 PTPases do not show a discernible tendency to transfer the PO_3 group to ethylene glycol (at up to 4 M concentration) or to glycerol, as assayed either by the ratio of PNP/P_i in the presence of alcohols or by specific search for formation of glycerol phosphate by ^{31}P NMR. Similarly, the 90-kDa CD45 catalytic fragment displayed no phosphotransferase activity to alcohols as cosubstrates.

The ability of LAR-D1 or LAR-D1D2 PTPase to catalyze a partial back-reaction, P_i to Enz·P_i to Enz–X–PO_3^-, could be readily assessed by incubation of $P^{18}O_4^-$ with enzyme in $H_2{}^{16}O$. In the case of the low molecular weight PTPase from beef heart, Van Etten and colleagues observed an exchange with a k_{cat} of 1.23×10^{-3} s^{-1} at pH 5 and 25 °C (Zhang & Van-Etten, 1991). Figure 2A shows the ^{31}P NMR spectrum of the ^{18}O-containing inorganic phosphate at t = 0, 30, 80, and 174 h after addition of 1.1 mg of pure LAR-D1D2. Clearly, the LAR enzyme catalyzes exchange not detected in the absence of PTPase (Figure 2B). From the exchange progress, a k_{cat} of 1.1×10^{-2} s^{-1} can be calculated, 9-fold faster than the beef heart PTPase. From integration of the phosphorus peaks in the NMR, it is possible to determine the partition ratio of a P_i molecule bound to the active site of LAR-D1D2 between reaction to form the proposed Enz–X–PO_3^{2-} and release back to solvent. The ratio was ~0 for LAR-D1D2 and 0.08 for LAR-D1. Even though the exchange reaction is quite slow with regard to the forward hydrolysis rate, LAR-D1(D2) PTPase is able to make a phosphoenzyme reversibly from P_i product. At 174 h (Figure 2A), each LAR-D1D2 molecule had catalyzed about 7000 exchange events (51 mM P_i, 25 μM enzyme). An almost equivalent ^{18}O-exchange rate was detected with LAR-D1 in a separate experiment.

Although the high k_{cat} values (and the lack of detectable phosphoryl transfer to alcohols) for phosphotyrosyl peptide hydrolysis by LAR-D1, LAR-D1D2, and CD45 (1000–3000 min^{-1}) suggested it might be difficult to accumulate a significant amount of Enz–X–PO_3^{2-} in turnover, we turned to phospho-EGFR peptide labeled with ^{32}P in an initial search for formation of ^{32}P-labeled PTPases in short incubation ex-

Scheme I



periments. To that end, the ^{32}P-EGFR peptide was prepared at a specific activity of 1250–5000 Ci/mmol from [γ-^{32}P]ATP and EGFR peptide with partially purified p56^{lck} tyrosine kinase expressed in and purified from baculovirus-infected SF9 cells (Ramer et al., 1991). The ^{32}P-EGFR peptide was purified from unreacted [γ-^{32}P]ATP by HPLC separation and then incubated with LAR or CD45 PTPase fragments for 5 or 10 s at 4 °C before rapid denaturation with SDS sample buffer followed by gel electrophoresis and autoradiography. In these studies with PTPase fragments, we used a substrate to enzyme ratio of 10/1 or 20/1, and labeling of all three PTPase fragments was detected (data not shown). For trial assessment of turnover of such a ^{32}P-enzyme species, the LAR-D1 fragment was incubated as described under Materials and Methods at an S–$^{32}PO_3$/E ratio (S, substrate; E, enzyme) of 10/1 for 10 s and quenched as above (lane B of Figure 1, bottom) or diluted at 20 s with a 3.3-fold molar excess of nonradioactive ^{31}P-EGFR peptide and chased for 20 min before being quenched with SDS and gel analysis (lane C of Figure 1, bottom). Clearly, the ^{32}P label is lost from the enzyme in the chase conditions. As a control, no labeled enzyme was detected if the PTPase fragment was first denatured by heating at 95 °C before addition of ^{32}P-tyrosyl peptide substrate. The ^{32}P-enzyme in lane B of Figure 1 (bottom panel) combined 900 cpm of ^{32}P, corresponding to a fractional stoichiometry of 4 phosphoryl groups per 1000 enzyme molecules.

Discussion

The purification of recombinant cytoplasmic catalytic domains of the human transmembrane PTPases LAR and CD45 from *E. coli* expression systems permits initial characterization for catalytic competence, specificity, and mechanism. A direct comparison of LAR-D1 vs LAR-D1D2 is consistent with previous mutagenesis studies which show that only the D1 domain was functional (Tsai et al., 1991; Streuli et al., 1990). Here we show the tandem second D2 domain provides no alteration of turnover number of specificity with five phosphotyrosyl peptides corresponding to autophosphorylation sequences of probable PTPase substrates.

The second clear conclusion about recognition and processing of phosphotyrosyl peptide substrates is that the cytoplasmic domains of LAR and CD45 show no real distinctions among the substrates dephosphorylated. Both catalytic domains are highly efficient enzymes with turnover numbers of 15–60 s^{-1} that are intrinsically fast and 10–100-fold faster than the protein tyrosine kinases with which they are balanced for signaling control (Ramer et al., 1991; Cantley et al., 1991). It will require both pure full-length transmembrane PTPases and identification of particular extracellular ligands for these receptor PTPases to determine whether k_{cat} and k_{cat}/K_m catalytic efficiency values are dialed down (they cannot be dialed very far up from the 10^6–10^7 M^{-1} s^{-1} values) on specific extracellular signaling as a mechanism for signal transduction.

Given the parallel specificity profile for the LAR and CD45 PTPase catalytic domains, and if that continues for other PTPases being isolated, it is likely that any functional specificity imposed in vivo between a given PTPase and its substrates such as phosphorylated protein tyrosine kinase molecules may come from propinquity of intracellular domains possibly arising by cocapping of external domains for example. Lessened promiscuity of phosphotyrosine sequence recognition may ensue also if full-length pY-containing proteins either alone or in complex with partner proteins are examined as PTPase substrates.

Alkaline and acid phosphatases use enzyme nucleophilic side chains (serine, histidine) to attack phosphomonoester substrates and yield covalent phosphoenzymes that then undergo hydrolysis (Coleman & Gettins, 1983; Van Etten & Hickey, 1977). In an initial assessment of phosphoenzyme formation by LAR-D1 or CD45-D1D2 90-kDa fragment, no phosphoryl group transfer to alcohols as cosubstrate acceptors was detectable nor was there a substantial fraction of enzyme accumulating as phosphoenzyme intermediate in turnover. However, analysis of the ability to catalyze reversible phosphoenzyme formation from the back direction could be conveniently probed by the technique of "washout" of oxygen-18 isotope from $P^{18}O_4$ into solvent as assessed by changes in the ^{31}P NMR spectrum of inorganic phosphate. While in a formal sense this only proves the enzyme's ability to catalyze a partial reaction, this P_i/H_2O exchange is a characteristic manifestation of enzymatic catalysis involving phosphoenzyme intermediates including ATPases (Faller & Diaz, 1989), alkaline phosphatases (Eargle et al., 1977), and most notably the low molecular weight PTPase that Van Etten and colleagues have recently shown by other criteria to proceed via an isolable E–X–PO_3^{2-} intermediate (Zhang & VanEtten, 1991). The LAR-mediated ^{18}O exchange rate while slow is in fact 9-fold faster than that observed with the low molecular weight PTPase. These results suggest the two-step mechanism of Scheme I where the ^{18}O exchange specifically monitors reversible progress of step 2 and E–X–PO^{32-} from the back direction. To search for the E–X–PO_3^{2-} intermediate from the forward direction (step 1), initial indications of ^{32}P-enzyme forms in dephosphorylative turnover of a $^{32}PO_4$-EGFR peptide have been obtained in this work by 10-s incubation at 4 °C followed by SDS gel analysis. The autoradiograms of Figure 1 (bottom) indicate ^{32}P label into LAR-D1. The initial pulse/chase results are consistent with a labile phosphoenzyme as anticipated for a hydrolase, but will require optimization to quantitate the accumulating fraction of phosphoenzyme and the site of phosphorylation as well as kinetic analysis to determine if the [^{32}P]phosphoryl group is indeed on the hydrolytic pathway. These studies will require a ^{32}P-Tyr peptide substrate with a low micromolar K_m value to achieve saturation at reasonable S/E levels and rapid-quench single-turnover studies under conditions where the denatured E–PO_3 species is stable. This will allow determination of conditions for accumulation of sufficient phosphoprotein for spectroscopic characterization of chemical structure. Independent detection of phosphoenzyme species via similar preliminary ^{32}P-labeling experiments has been reported (Dixon et al., 1991). It also remains to be seen whether the essential and highly conserved cysteine (Cys-1522 in LAR) in this class of receptor PTPases has any role in catalysis as the catalytic nucleophile to yield a Cys–S–PO_3^{2-} intermediate (Dixon et al., 1991).

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Interpretation of the Effect of an Oscillating Electric Field on Membrane Enzymes

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ABSTRACT: Theoretical expressions for the frequency and amplitude dependence of the rate of a catalyzed reaction are fitted to the data of Graziana et al. (1990) [Graziana, A., Ranjeva, R., & Teissié, J. (1990) *Biochemistry 29*, 8313-8318] for Ca^{2+} uptake by carrot protoplasts in an oscillating electric field. This uptake is a direct (linear) measure of the rate of increase of ATP caused by a plasma membrane enzyme in the oscillating field. The fit gives 20 ms and 33 μs for the relaxation times of the enzyme and roughly 3 for the effective number of elementary changes displaced across the membrane by a conformational change of the enzyme in its catalytic cycle. Additional experiments are suggested to define further the mechanism of the enzymatic reaction.

Graziana et al. (1990) have observed that an applied 100-Hz oscillating electric field of 25 V/cm peak amplitude stimulates Ca^{2+} uptake by carrot protoplasts by as much as a factor of 3. The Ca^{2+} influx increased with electric treatment duration up to 5 min and then decreased slowly to approximately its initial value after 15 min. Remarkably, the stimulation elicited by the electric field was still effective after the field was switched off. After an initial 5-min treatment was turned off, the influx dramatically continued to increase for 5 min and then decreased slowly. The Ca^{2+} influx after 5-min treatment also was found to depend on the oscillating field amplitude and frequency. Thus, the effect could not be due to joule heating, which is independent of frequency. During electric stimulation, the amount of internal ATP increased by more than 30%. After the field was turned off, the extra amount of ATP was almost completely consumed within 5 min, corresponding to the time of maximum influx of Ca^{2+}. Addition of 50 μM dicyclohexylcarbodiimide (DCCD), an inhibitor of ATP synthesis, suppressed all the electric field induced effects, i.e., the increase in ATP concentration and Ca^{2+} influx. The authors conclude that the oscillating electric field stimulates an increase in ATP that drives the influx of Ca^{2+}.

They report that for a constant-amplitude and constant-frequency field applied for up to 5 min both the rate of Ca^{2+} influx and the amount of additional ATP are proportional to the time of application of the oscillating field. Thus, their amplitude- and frequency-dependent Ca^{2+} influx measurements (which were obtained by applying a steady oscillating field for 5 min) are proportional to the amplitude- and frequency-dependent amount of additional ATP.

Transmembrane proteins respond to applied electric fields (Witt et al., 1976; Teissié et al., 1981; Teissié, 1986) because (1) the field is strongly concentrated in the plasma membrane, (2) the conformational changes of many membrane proteins involve a large displacement charge, and (3) the membrane prevents the protein from rotating and thereby evading the effect of the field (Tsong & Astumian, 1986).

Membrane enzymes undergo conformational changes in their catalytic cycle. The motion of charge during this conformational change provides a coupling between an electric