Purification and Characterization of a Soluble Catalytic Fragment of the Human Transmembrane Leukocyte Antigen Related (LAR) Protein Tyrosine Phosphatase from an *Escherichia coli* Expression System†

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ABSTRACT: A 350 amino acid soluble fragment of the intracellular catalytic domain of the human transmembrane leukocyte antigen related (LAR) protein tyrosine phosphatase has been purified 17-fold to >90% purity from an *Escherichia coli* expression vector in quantities sufficient for kinetic and structural characterization. To assess substrate specificity, phosphotyrosine peptides corresponding to autophosphorylation sites of the two major classes of tyrosine kinases have been synthesized. Thus 6–12-residue phosphotyrosine peptides of the insulin receptor and epidermal growth factor receptor kinase domains and of the autophosphorylation and C-terminal regulatory sites of $p60^{src}$ and $p56^{lck}$ have been analyzed for k_{cat} and K_M by using a nonradioactive chromogenic assay for P_i release. The catalytic domain of LAR PTPase shows k_{cat} values of 20–70 s^{-1} for phosphotyrosine peptides and affinities that vary 150-fold from 27 μM to 4.1 mM.

Much attention has been directed to the study of enzymes with protein tyrosine kinase activity in view of their central role in signal transmission from cell-membrane initiated ligand binding and also due to the oncogenic consequences of overexpression or excess activity of tyrosine kinases [for reviews see Hunter and Cooper (1986), Jove and Hanafusa (1987), Ullrich and Schlessinger (1990), and Yarden and Ullrich (1988)]. Both receptor transmembrane tyrosine kinases and the *src* family of myristoylated peripherally associated membrane tyrosine kinases have been analyzed genetically and biochemically [for reviews see Hanks et al. (1988), Hunter (1987), and Kemp (1990)]. The phosphorylation of protein tyrosyl residues is likewise implicated in molecular controls for cell cycle transitions (Gould et al., 1990; Gould & Nurse, 1989). The use of tyrosine phosphorylations in specific proteins to transmit signals must be balanced by a complementary cadre of protein tyrosine phosphatases to ensure the covalent phosphorylation events are reversible and to oppose and control the extent and duration of the signals transmitted. The net moment to moment readout is a balance of protein tyrosine kinase and protein tyrosine phosphatase action.

While molecular characterization of the phosphatases has lagged behind that of the tyrosine kinases, the purification of a soluble placental PTPase 1B (Tonks et al., 1988b) leading to the recognition of a homologous domain in the transmembrane CD45 (LCA)[1] leukocyte protein (Charbonneau et al., 1988, 1989) and establishment of PTPase activity of CD45 (Tonks et al., 1988a) has accelerated the pace substantially. Now some 10 eukaryotic membrane-linked PTPases have been cloned and sequenced (Cool et al., 1989; Guan et al., 1990; Krueger et al., 1990; Streuli et al., 1988, 1989), among them the widely distributed LAR (leukocyte antigen related) PTPase that has been expressed in *Escherichia coli* and subjected to mutagenesis to determine functional domains and amino acid residues (Streuli et al., 1990).

We report here the purification and characterization of the 350 amino acid containing catalytic domain of LAR PTPase as a soluble fragment from an *E. coli* expression system and its catalytic characterization with a series of specific phosphotyrosyl peptides corresponding to the phosphorylation sites of tyrosine kinases.

Materials and Methods

General. Fmoc-Leu-OH, Fmoc-Lys(Boc)-OH, Fmoc-Arg(Pmc)-OH, Fmoc-Tyr(Bu^t)-OH, Fmoc-Asp(Bu^t)-OH, Fmoc-Thr(Bu^t)-OH, Fmoc-Glu(Bu^t)-OH, Fmoc-Ile-OH, Fmoc-Ala-OH, Fmoc-Val-OH, Fmoc-Gln(Trt)-OH, Fmoc-Asn(Trt)-OH, and HMP resin 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 by using the Bio-Rad dye reagent (cat. no. 500-0006) following the

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[1] Abbreviations: LCA, leukocyte common antigen; BOP, benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorphosphate; HOBT 1-hydroxybenzotriazole hydrate; TPTU, 2-(2-oxo-1(2H)-pyridyl)-1,1,3,3-tetramethyluronium tetrafluoroborate; HBTU, 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; LAR, leukocyte antigen related; PTPase, protein tyrosine phosphatase; Fmoc, fluorenylmethoxycarbonyl; Maq, 2-oxymethyleneanthraquinone; RP-HPLC, reverse-phase high-pressure liquid chromatography; MES, 2-(*N*-morpholino)ethansulfonic acid; HEPES, *N*-(2-hydroxyethyl)-piperazine-*N*′-(2-ethanesulfonic acid); EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; TFA, trifluoroacetic acid; IR, insulin receptor; EGFR, epidermal growth factor receptor.

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manufacturers directions with bovine serum albumin as the standard.

Expression of the Active Catalytic Domain of LAR (LAR-D1) PTPase in E. coli. In order to overproduce LAR-D1 PTPase, a prokaryotic expression vector pT7-7 that contains a strong promoter for T7 RNA polymerase (Tabor & Richardson, 1985) and an *E. coli* strain BL21(DE3) that carries a single chromosomally integrated copy of the T7 polymerase gene under the control of the inducible *lac*UV5 promoter (Studier & Moffatt, 1986) were utilized. Addition of isopropyl β-D-thiogalactoside induces the production of T7 RNA polymerase in BL21(DE3), which in turn induces transcription of a sequence on the pT7-7 plasmid under the control of the T7 promoter.

The segment of the human LAR PTPase cDNA encoding the amino acid positions 1275–1613 was modified so that this sequence is flanked by a *Bam*H1 site at the 5′-end and a termination codon and a *Hin*dIII site at the 3′-end. The *Bam*HI–*Hin*dIII fragment thus generated was inserted between the *Bam*HI and *Hin*dIII sites within the polylinker sequence in the pT7-7 prokaryotic expression vector. Thus, 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.

Purification of LAR-D1 PTPase. Isopropyl β-D-thiogalactoside (final concentration, 0.4 mM) and 10% glucose (final concentration, 0.4%) were added to exponentially growing cultures of *E. coli* BL21 (DE3) carrying the pT7-7 LAR-D1 plasmid in LB medium containing ampicillin (100 μg/mL). Cultures (800 mL) were shaken at 30 °C for 3 h, and cells were collected by centrifuging at 4000 rpm (Beckman J-6) for 20 min at 4 °C. The pellet was resuspended in 9 mL of buffer A (33 mM Tris·HCl, 2.5 mM EDTA, 10 mM 2-mercaptoethanol, pH 8.0) and transferred to Sorval T-865 centrifuge tubes. Lysozyme (5 mg) in 1 mL of buffer A was added, and the suspension was incubated at room temperature for 15 min followed by freezing in dry ice/ethanol. After being thawed at 25 °C (~10 min) this was frozen and thawed for two more cycles. The resulting lysate was incubated with 100 μL of 1 M $MgCl_2$ and 100 μL of DNase (5 mg/mL in buffer A) for 30 min at 25 °C. After the addition of 0.4 mL of 0.5 M EDTA and 1.2 mL of 10% Triton X-100, the mixture was incubated at 25 °C for 10 min. This was centrifuged at 30000 rpm (Sorval T-865) for 30 min at 4 °C. The supernatant (~25 mL) was fractionated with ammonium sulfate, and the 25–50% fraction was dialyzed against buffer A containing 30% glycerol prior to being stored frozen at −80 °C.

The 25–50% ammonium sulfate fraction (2.2 mL) was thawed, diluted with 2.0 mL of buffer B (10 mM imidazole·HCl, 2 mM EDTA, 0.1% 2-mercaptoethanol, 1 mM benzamidine, pH 7.2), and applied to a DEAE-cellulose column (1.4 cm × 4 cm) equilibrated with buffer B. After the sample was loaded by gravity, the column was washed with 10 mL of buffer B containing 0.02 M NaCl. The protein was eluted with buffer B containing 0.05 M NaCl, and the active fractions (as determined by the appearance of a yellow color upon addition to 100 mM *p*-nitrophenyl phosphate in buffer A) were combined and concentrated (Amicon PM-10) to ~3 mg/mL protein. Glycerol was added to a final concentration of 30% before storing frozen at −80 °C. LAR phosphotyrosine phosphatase activity was determined by measuring the release of inorganic phosphate from the phosphotyrosine containing peptide $p60^{src}_{523-531}$ (see below).

Synthesis of Fmoc-Tyr(PO_3Me_2)-OH. A previous outlined procedure was used for the synthesis of this synthon (Kitas et al., 1989). Briefly, this involved the two-step phosphite triester phosphorylation of Fmoc-Tyr-OMaq using $Et_2NP(OMe)_2$ as a phosphinylating reagent in the presence of 1H-tetrazole (Bannwarth & Trzeciak, 1987; Kitas et al., 1990b; Perich & Johns, 1988) followed by oxidation with *m*-chloroperbenzoic acid. From the resulting Fmoc-Tyr(PO_3Me_2)-OMaq, the Maq protecting group was removed by reduction with sodium dithionite (Hoogerhout et al., 1985). Furthermore, a one-pot phosphorylation of Fmoc-Tyr-OH has also been developed (unpublished results). This procedure involves in situ carboxyl protection of Fmoc-Tyr-OH with *tert*-butyldimethylsilyl chloride, followed by the 1H-tetrazole catalyzed phosphite triester phosphorylation, oxidation to the phosphotriester, and aqueous acid treatment to cleave the silyl ester. Such a one-pot procedure has been previously reported for the synthesis of Boc-Tyr(PO_3Me_2)-OH (Perich & Johns, 1989).

Synthesis of Phosphotyrosine-Containing Peptides. The phosphorylated epidermal growth factor receptor (EGFR) and insulin receptor (IR) peptides (Table II) were prepared by Fmoc solid-phase synthesis using HMP resin and BOP/HOBT chemistry (Hudson, 1988) on a 0.15-mmol scale with a Milligen 504 shaker. Phosphorylated and nonphosphorylated peptides of equivalent sequences were synthesized in parallel with the resin being divided at the divergent residue (i.e., phosphotyrosine). The standard coupling conditions used 3 equiv of the protected amino acid. However, to conserve Fmoc-Tyr(PO_3Me_2)-OH, 1.1 equiv was used, and, after shaking for 3 h, the unreacted amino functional groups were blocked with 10% acetic anhydride in *N*-methylpyrrolidone. Couplings after the addition of the protected phosphotyrosine required extended reaction times (2–6 h) and were monitored by ninhydrin analysis of the resin.

Alternatively, the phosphorylated $p60^{src}$ and $p56^{lck}$ peptides (Table II) were prepared by continuous flow variation of the Fmoc method of solid-phase synthesis (Dryland & Sheppard, 1986) on a Milligen 9050 automated synthesizer using either HBTU or TPTU activation (Knorr et al., 1989). An excess of 2.5 equiv of all amino acids was applied in conjunction with 30-min acylation times. All three syntheses proceeded smoothly as judged by on-line UV monitoring and RP-HPLC analysis of the crude product mixture following deprotection. No acetic anhydride capping was used in these syntheses.

Deprotection, including removal of the phosphate protecting groups, and cleavage from the resin of phosphotyrosine containing peptides was accomplished with a mixture of trimethylsilyl bromide (0.4 mL), thioanisole (0.35 mL), and *m*-cresol (0.05 mL) in TFA (2.2 mL) as previously described (Kitas et al., 1989). The resulting crude peptides were purified by reverse-phase HPLC chromatography on a Bio-Rad 21.5 mm × 250 mm C_{18} column with a linear gradient from 0.01% TFA in water to 0.01% TFA in CH_3CN at a flow rate of 10 mL/min.

Assay for Tyrosine Phosphatase Activity. To assay the dephosphorylation of phosphotyrosine containing peptides by LAR PTPase, the release of inorganic phosphate was determined with the malachite green assay (Lanzetta et al., 1979; Martin et al., 1985). For this assay, the enzyme was diluted with buffer (25 mM HEPES, 5 mM EDTA, 10 mM DTT, 1 mg/mL bovine serum albumin, pH 7.3) and added to the reaction mixture containing the phosphopeptide in buffer (100 mM MES, 5 mM EDTA, 10 mM DTT, pH 6.0). 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

Table I: Purification of LAR-D1 PTPase from 800 mL of *E. coli* Culture

	vol (mL)	total protein (mg)	sp act. (μmol min^{-1} mg^{-1})[a]	total act. (μmol min^{-1})	yield (%)
lysate	24	151	1.8	272	100
$(NH_4)_2SO_4$	23	71	2.9	206	77
DEAE	0.68	2.7	31	84	34

[a] The micromoles of inorganic phosphate released per minute from p60 $^{srcpY527}_{523-531}$ (0.83 mM) at 25 °C by 1 mg of protein.

800 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.

Results

LAR PTPase Catalytic Domain Purification. In a previous study of the effects of mutations on the intracellular catalytic domain of the LAR protein tyrosine phosphatase, the activity of a 339-residue segment, corresponding to residues 1275–1613 of full-length LAR PTPase, has been assessed in crude extracts of an *E. coli* overproducing lysate by assaying the release of ^{32}P label from ^{32}P-phosphorylated Raytide or phosphorylated myelin basic protein (Streuli et al., 1989, 1990) containing ^{32}P at a minor fraction of tyrosine residues in these substrates. To obtain the LAR PTPase fragment as a 350 amino acid fragment (see Materials and Methods) in greater than 90% purity (as judged by densitometry scanning of Coomassie blue stained SDS-PAGE gels) (Figure 1), the enzyme was subjected to ammonium sulfate fractionation and DEAE ion-exchange chromatography resulting in a 17-fold purification (Table I). The purified enzyme can be stored in 30% glycerol at −80 °C for months without substantial activity loss, but a significant decrease in activity is observed with repeated freeze–thawing. Also, on dilution to the 0.4–6 μg/mL concentrations of protein used in enzymatic assays, variable loss of activity was detected; this could be prevented by inclusion of bovine serum albumin (1 mg/mL) in the enzyme dilution buffer. Typically 3.4 mg of pure 40-kDa catalytic LAR PTPase domain are available from 1 L of cell growth. Validation that intact LAR PTPase domain was purified came from determination of 15 residues of the N-terminus that yielded the sequence ARIRARGSGLKDSLL in agreement with the predicted sequence minus the N-terminal methionine that has apparently been cleaved by the *E. coli* methionyl peptidase.

Preparation and Purification of Phosphotyrosyl Peptides. One approach to evaluate whether the catalytic domain of LAR PTPase recognizes particular phosphotyrosine-containing proteins as substrates with differential affinity is to prepare

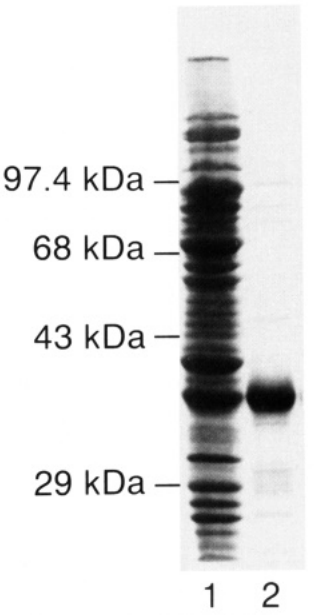

FIGURE 1: SDS–PAGE analysis of LAR PTPase purification; ammonium sulfate fraction (lane 1) and DEAE column fraction (lane 2). The proteins were separated on a 10% acrylamide gel and stained with Coomassie blue.

specific phosphotyrosine peptides corresponding to known substrates of PTPases. While in many cases the cellular proteins phosphorylated at tyrosines are still poorly identified, the tyrosine kinases of both the growth factor receptor type (Bertics et al., 1988; Downward et al., 1984; Rosen, 1987; White et al., 1988) and of the *src* kinase family are known to undergo tyrosyl autophosphorylation that alters kinase activity (Hunter, 1987). In turn, these phosphotyrosine sequences undergo dephosphorylation by one or more PTPases of which the widely expressed LAR PTPase is a candidate. Therefore we prepared phosphotyrosine peptides (6–12 amino acids) corresponding to the autophosphorylation sites of the EGF receptor kinase (residues 1167–1177) and of the insulin receptor kinase (residues 1142–1153) [see Kemp and Pearson (1990) and references therein]. In the case of the insulin receptor kinase, evidence exists that each of the three tyrosine residues are autophosphorylated (White et al., 1988), so a phosphotyrosine residue was incorporated at the 5th, 9th, or 10th residues (corresponding to tyrosines 1146, 1150, and 1151) to yield the three IR peptides ($IR^{pY1146}_{1142-1153}$, $IR^{pY1150}_{1142-1153}$, and $IR^{pY1151}_{1142-1153}$) of Table II. The *src* family recognition was probed with a phosphotyrosine peptide from the known autophosphorylation sites in $p60^{c\text{-}src}$ (Y416) and $p56^{lck}$ (Y394) ($p56^{lckpY394}_{390-398}$) as well as peptides from a C-terminal tyrosine site (505 in $p56^{lck}$, $p56^{lckpY505}_{501-509}$ and 527 in $p60^{c\text{-}src}$, $p60^{srcpY527}_{523-531}$) whose phosphorylation down regulates these tyrosine kinase activities [for reviews see Bolen and Veillette (1989), Gallagher

Table II: Summary of the Kinetic Constants for the Dephosphorylation of Phosphotyrosyl Peptides by LAR-D1 PTPase

		K_M (mM)	V_{max} (μmol min^{-1} mg^{-1})	k_{cat} (s^{-1})	k_{cat}/K_M (s^{-1} M^{-1})
AEpYLRV	$EGFR^{pY1173}_{171-1176}$	4.1 ± 0.7	104 ± 21	69	1.7×10^4
TAENAEpYLRVA	$EGFR^{pY1173}_{167-1177}$	0.48 ± 0.05	68 ± 3	45	9.4×10^4
TRDIpYETDYYRK	$IR^{pY1146}_{1142-1153}$	0.027 ± 0.001	96 ± 1	64	2.4×10^6
TRDIYETDpYYRK	$IR^{pY1150}_{1142-1153}$	0.28 ± 0.03	33 ± 1	22	7.8×10^4
TRDIYETDYpYRK	$IR^{pY1151}_{1142-1153}$	0.71 ± 0.18	45 ± 4	30	4.2×10^4
EDNEpYTARE	$p56^{lckpY394}_{390-398}$	0.17 ± 0.05	75 ± 3	50	2.9×10^5
TEGQpYQPQP	$p56^{lckpY505}_{501-509}$	0.73 ± 0.1	27 ± 9	18	2.5×10^4
TEPQpYQPGE	$p60^{srcpY527}_{523-531}$	0.35 ± 0.09	58 ± 8	38	1.1×10^5
phosphotyrosine		>6	>30		
phosphoserine			<0.001		
phosphothreonine			<0.001		

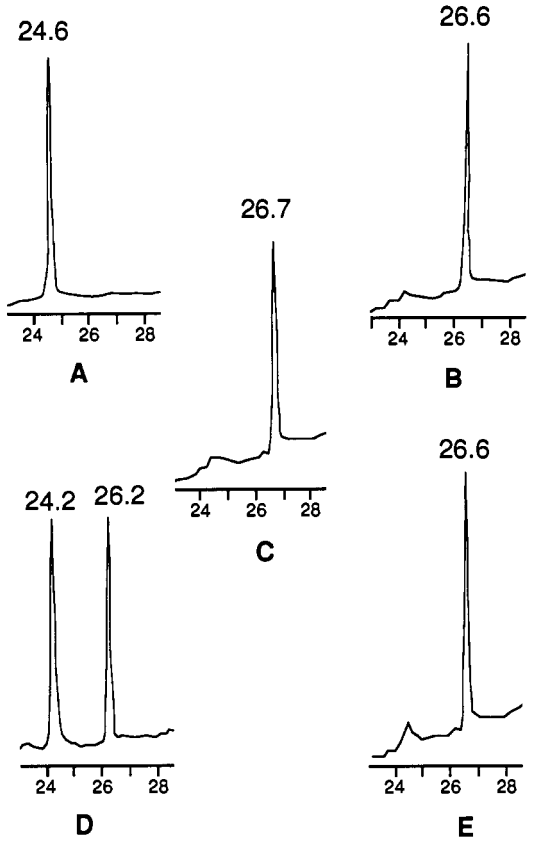

FIGURE 2: Verification of the identity of phosphotyrosine peptide $IR^{pY1146}_{1142-1153}$ by complete dephosphorylation by LAR PTPase and analysis by HPLC. Authentic $IR^{pY1146}_{1142-1153}$ (A) was incubated with LAR PTPase (0.1 μg) at 25 °C for 120 min prior to injection onto HPLC to obtain trace C. Trace B is the authentic $IR_{1142-1153}$ (unphosphorylated peptide), trace D is from mixing A and C, and trace E is from mixing B and C. Results show that the product of the enzymatic dephosphorylation coelutes with unphosphorylated $IR_{1142-1153}$ (retention time ~26 min), distinct from the phosphorylated $IR^{pY1146}_{1142-1153}$ (retention time ~24 min). HPLC was done with a Vydac C_{18} 4.6 cm × 25 cm HPLC column with a gradient from TFA (0.1%) in water to 30% acetonitrile (0.1% TFA) over 30 min at a flow rate of 1 mL/min.

and Cambier (1990), and Hunter (1987)].

Batch-wise solid-phase synthesis of the phosphotyrosine-containing peptides using Fmoc-Tyr(PO_3Me_2)-OH and BOP/HOBT chemistry proceeded sluggishly, requiring extended reaction times for the incorporation of the phosphotyrosine synthon and all the subsequent acylations. Blocking of unreacted amino acid functional groups was necessary to facilitate purification of the final peptides. Conversely, with the application of continuous flow variation and TPTU or HBTU chemistry, excellent results were obtained. RP-HPLC analysis of the peptides cleaved with 1 M trimethylsilyl bromide–PhSMe/TFA (*m*-cresol) showed predominantly one product.

Validation that the HPLC-purified synthetic products were the desired phosphotyrosine peptides was obtained in four ways. As shown in Figure 2 for the $IR^{pY1146}_{1142-1153}$ peptide, incubation with pure LAR PTPase catalytic domain converted the starting phosphotyrosine peptide to material with the HPLC mobility of authentic unphosphorylated peptide made by separate synthesis. Similar results were obtained for all the peptides synthesized. Second, the phosphotyrosine-containing peptides were analyzed by capillary zone electrophoresis. Third, fast atom bombardment mass spectroscopic analysis confirmed the molecular weight for each phosphotyrosine peptide and gave fragments consistent with sequence.

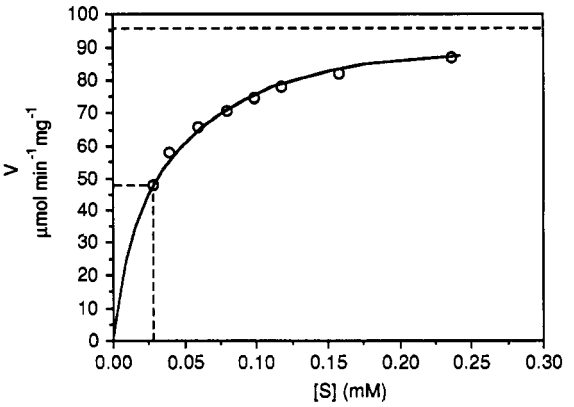

FIGURE 3: A typical V vs [S] curve with the malachite green assay for LAR PTPase dephosphorylation of $IR^{pY1146}_{1142-1153}$ from which V_{max} and K_M values were determined by fitting to a hyperbola. Data were obtained under conditions where <20% of the phosphopeptide was converted to product to ensure that initial velocities were obtained.

Fourth, the phosphopeptides were sequenced by Edman degradation where the phosphotyrosine residues are identified as a blank in the analysis (Kitas et al., 1989).

Catalytic Properties of LAR PTPase Catalytic Domain with Phosphotyrosine Peptides. The availability of pure phosphotyrosine peptides of known sequence and stoichiometry permit the assay of PTPases including the LAR PTPase catalytic domain under V_{max} conditions so that k_{cat} and K_M values can be readily obtained and compared. Most prior assays have used ^{32}P-labeled phosphorylated tyrosine containing peptides such as Raytide (modified gastrin), angiotensin, or phosphorylated forms of myelin basic protein and reduced carboxymethylated lysozyme (Streuli et al., 1989, 1990; Tonks et al., 1988c, 1990). These substrates are only partially phosphorylated by the conditions under which they are prepared, and, for large peptides and proteins with more than one site of phosphorylation, mixtures of differentially phosphorylated products may be obtained. Furthermore, only limited quantities of phosphorylated material can be prepared. These factors limit the interpretation of the results obtained with these substrates with PTPases. To overcome these limitations, we have used chemical synthesis of phosphotyrosyl peptides for assays of PTPase activity. We further wished to avoid the need to prepare each specific phosphotyrosine peptide in radiolabeled form and have adapted the malachite green colorimetric assay for the detection of inorganic phosphate (Lanzetta et al., 1979), previously used by Graves and coworkers (Martin et al., 1985), among others, in assay of the dephosphorylation by calcineurin of the amino acid phosphotyrosine. The sensitivity is from 1 to 50 nmol P_i (Lanzetta et al., 1979). Figure 3 shows a typical V vs [S] progress curve for the LAR PTPase processing of the $IR^{pY1146}_{1142-1153}$ peptide.

All three types of phosphotyrosine peptides corresponding to IR and EGFR autophosphorylation sites, to $p60^{c\text{-}src}$ and $p56^{lck}$ autophosphorylation sites, and to the C-terminal negative regulatory phosphotyrosine sites of $p60^{c\text{-}src}$ and $p56^{lck}$ are dephosphorylated by the pure LAR PTPase catalytic domain (Table II). The highest V_{max} of Table II of 104 μmol min^{-1} mg^{-1} for $EGFR^{pY1173}_{1171-1176}$, given the molecular weight of 40000 of the LAR PTPase domain, converts to a k_{cat} of 69 dephosphorylation events per second, a rapid catalyst.

The peptides corresponding to autophosphorylation sites in the EGFR and IR proteins show distinct behavior with LAR PTPase. The hexapeptide $EGFR^{pY1173}_{1171-1176}$ is a high k_{cat} (69 s^{-1}) and high K_M substrate (4.1 mM); however, increasing the length of the peptide to 11 amino acids ($EGFR^{pY1173}_{1167-1177}$) lowered the K_M substantially to 0.48 mM and decreased the k_{cat}

to 45 s^{-1}. The addition to the hexapeptide of five amino acids, four at the N-terminus and one at the C-terminus, therefore increases the catalytic efficiency of LAR PTPase for this sequence 5-fold.

The insulin receptor peptides corresponding to residues 1142–1153 encompasses three tyrosines at 1146, 1150, and 1151 known to be autophosphorylated in vivo, such that di- and triphospho forms accumulate (White et al., 1988). As a first approach to evaluate the ability of the LAR PTPase catalytic domain to distinguish phosphotyrosyl residues at each of the three sites, we synthesized and purified each monophosphotyrosyl regioisomer, designated $IR^{pY1146}_{1142-1153}$, $IR^{pY1150}_{1142-1153}$, and $IR^{pY1151}_{1142-1153}$. Most striking is the wide 30-fold range in K_M values from 27 to 710 μM. The 27 μM K_M value for the $IR^{pY1146}_{1142-1153}$ peptide demonstrates that relatively short peptides with phosphotyrosyl residues can be high-affinity substrates. The phosphotyrosyl residue is crucial for phosphatase recognition since the dephosphorylated 12-mer of the same $IR_{1142-1153}$ sequence showed no detectable inhibition (data not shown). The k_{cat}/K_M ratio for $IR^{pY1146}_{1142-1153}$ of 2.4×10^6 M^{-1} s^{-1} reflects a very high catalytic efficiency for LAR PTPase domain as a catalyst. The $IR^{pY1150}_{1142-1153}$ and $IR^{pY1151}_{1142-1153}$ 12-mers show a 3-fold difference in K_M with $IR^{pY1151}_{1142-1153}$, the least good of the three regioisomeric monophosphotyrosyl peptides both by K_M and k_{cat}/K_M criteria.

The *src* family of tyrosine kinases, exemplified both by an autophosphorylation peptide substrate from the highly conserved "kinase" core of $p60^{src}$ and $p56^{lck}$ and by two phosphotyrosyl peptides corresponding to the distinct C-terminal regulatory sequences of $p60^{c\text{-}src}$ and $p56^{lck}$, also show interesting variations as LAR phosphatase substrates. The $p56^{lckpY394}_{390-398}$ nonapeptide with phosphotyrosine-394 differs from the homologous autophosphorylation site in $p60^{c\text{-}src}$ (phosphotyrosine-416) only by an E to Q change in the last residue of the peptide for $p60^{c\text{-}src}$. The $p56^{lckpY394}_{390-398}$ peptide has a reasonable K_M of 170 μM and substantial k_{cat} (50 s^{-1}). By comparison, the C-terminal phosphotyrosyl peptide $p60^{srcpY527}_{523-531}$ shows a 2-fold (350 μM) and $p56^{lckpY505}_{501-509}$ a 4.3-fold (730 μM) higher K_M, respectively, again evidence that LAR PTPase shows differential recognition of phosphotyrosyl residues in distinct primary sequence contexts. The lower K_M of the $p60^{c\text{-}src}$ ($p60^{srcpY527}_{523-531}$) compared to the $p56^{lck}$ ($p56^{lckpY505}_{501-509}$) C-terminal phosphotyrosyl peptide is in the right direction for selective recognition of the widely expressed $p60^{c\text{-}src}$ by the also widely expressed LAR PTPase (Streuli et al., 1988). In contrast, $p56^{lck}$ expression is restricted to cells of lymphoid lineage (Marth et al., 1985) and LAR PTPase is not expressed in peripheral leukocytes, so $p56^{lck}$ will not likely be a physiological substrate for LAR PTPase while $p60^{c\text{-}src}$ is a possible candidate.

LAR PTPase can also dephosphorylate the free amino acid phosphotyrosine as noted in Table II. The samples of commercial phosphotyrosine contained contaminating P_i even after reverse-phase HPLC, and the results in Table II are therefore only preliminary estimates to date but do indicate a K_M in the >6 mM range. There was no detectable hydrolysis of free phosphoserine or free phosphothreonine such that the enzyme shows at least 10^4-fold specificity for phosphotyrosyl group hydrolysis.

Analysis of the pH optimum of LAR PTPase with peptide $IR^{pY1146}_{1142-1153}$ (0.3 mM) indicated a plateau of activity in the pH range of 3.5–7.2 with a decline to 25% activity by pH 3.0, indicating an "acid" phosphatase type profile (data not shown). Previous results in crude extract had argued against the need for added divalent cations and were corroborated by no observed change in activity levels in 5 mM EDTA and by a failure of 1 mM Zn^{2+} or Mn^{2+} to affect the dephosphorylation of $p60^{srcpY527}_{523-531}$ (Table III). Assays for tightly bound stoichiometric metals have not yet been conducted. The PTPase inhibitor sodium orthovanadate also inhibited the dephosphorylation of the phosphotyrosyl peptide to ~20% of the full activity at 0.2 μM vanadate (Table III). Higher concentrations of the inhibitor failed to further inhibit LAR PTPase, with some apparent regain (to 44% of initial activity) at 1 mM vanadate. No diminution of activity was observed when DTT was omitted from the enzyme dilution buffer and the assay mixture (although the enzyme needs to be stored with thiols present). The unphosphorylated $p60^{src}_{523-531}$ nonapeptide had no inhibitory effect at concentrations up to 0.4 mM on dephosphorylation of the phosphotyrosyl form (at 0.23 mM) of the same peptide.

Table III: Effect of Additives on Dephosphorylation of $p60^{srcpY527}_{523-531}$ at (0.23 mM)

additive (mM)	% of control
$MnCl_2$	
(1)	108
$ZnCl_2$	
(1)	116
Na_3VO_4	
(0.1)	39
(0.2)	19
(0.3)	18
(0.5)	21
(0.7)	25
(1.0)	44
$p60^{src}_{523-531}$ (not phosphorylated)	
(0.4)	115

Discussion

A soluble catalytic domain of 40 kDa encompassing residues 1275–1613 of the human transmembrane LAR protein tyrosine phosphatase, a representative of the "receptor PTPases" (Hunter, 1989), has been expressed and purified from *E. coli* in quantities sufficient for kinetic and structural characterization. A variety of mutant forms of this protein (Tsai et al., 1991) can also be characterized in an analogous fashion. This catalytic domain of LAR PTPase is about equally homologous to catalytic domains of other "receptor PTPases" such as leukocyte lineage-specific CD45 (LCA) and to soluble PTPases such as PTPase 1B that studies on the LAR PTPase catalytic domain may provide information of general relevance to understanding of PTPase mechanism and specificity. While the 623-residue cytoplasmic domain of LAR PTPase (and of CD45 among others) has two tandem "catalytic" domains, previous mutagenesis studies have indicated that only the membrane proximal domain 1 was functional (Streuli et al., 1990). Hence that fragment has been expressed and purified here as a prototype for a "core" PTPase domain available for structure/function analyses. Results on specificity and efficiency will serve as a benchmark for subsequent studies on two-domain and full-length LAR PTPase to analyze for modulation of basal, unregulated activity.

In addition to the purification of the catalytic domain of LAR PTPase, a second major goal of this work has been the synthesis, purification, and availability of phosphotyrosine peptides of specific sequences found in known phosphotyrosyl protein substrates of PTPases. To that end phosphotyrosyl peptides from the EGF receptor and from the insulin receptor represent one major class of PTPase substrate, while phosphotyrosyl peptides from the autophosphorylation site and the C-terminal negative regulatory site of $p60^{c\text{-}src}$ and $p56^{lck}$ represent a second major class known to undergo dephosphory-

lation in vivo. These phosphotyrosyl peptides have permitted some useful conclusions about LAR PTPase as a catalyst and may lead to design of inhibitors specific for particular PTPases.

First, the data of Table II confirm that the LAR PTPase catalytic domain acts on various phosphotyrosyl peptides with k_{cat} values in a narrow range of 20–70 s^{-1}. These high turnovers are similar to the soluble 321-residue PTPase 1B enzyme (Tonks et al., 1988c) and to purified CD45 (Tonks et al., 1990) and suggest high catalytic flux if the PTPases are not regulated. The possibility of regulation of "receptor PTPase" action by binding of ligands to extracellular domains can be investigated with full-length LAR PTPase or CD45 when such ligands become identified. A second feature, consistent with previous in vivo data on PTPases (Ostergaard et al., 1989), is that LAR PTPase shows some substantial discrimination in molecular recognition for phosphotyrosyl functionality even in the context of nona- to dodecapeptides.

The substrates of Table II exhibit a 200-fold difference in K_M (27–4100 μM) with no reason to suspect an optimal K_M has yet been approached. Studies on CD45 with phosphorylated reduced carboxymethylated lysozyme, an artificial protein PTPase substrate, indicate a K_M value of 12 μM with k_{cat} similar to that of the LAR PTPase catalytic domain (Tonks et al., 1990). We note that if a phosphotyrosyl peptide substrate of 1 μM is found with a k_{cat} of 50 s^{-1} (the average in Table II), then k_{cat}/K_M will be 5×10^7 M^{-1} s^{-1}, approaching the efficiency limited by diffusion events and suggesting these enzymes may have evolved to keep phosphotyrosine levels low in cells.

The three monophosphorylated regioisomers of the insulin receptor autophosphorylation site peptide $IR_{1142-1153}$ have been particularly useful in revealing a clear discrimination by the LAR PTPase catalytic domain and give impetus to synthesis and purification of the tri- and diphosphotyrosyl versions of this 12-mer of the IR β-subunit since it appears the insulin receptor, on insulin ligation and activation, exists as the triphosphoryl pY_{1146}, pY_{1150}, and pY_{1151} form that is converted to diphosphoryl forms by PTPases (White et al., 1988). The K_M and k_{cat} values of those peptides for LAR PTPase could be quite distinct from the monophosphotyrosyl forms, given the 30-fold variation detected among the monophosphotyrosyl isomers in Table II. These results and the values for the dephosphorylation of the phosphotyrosyl EGFR peptides, suggest that phosphotyrosyl peptides of platelet-derived growth factor (PDGF) receptor and colony-stimulating factor 1 (CSF-1) receptor warrant synthesis and testing.

The phosphotyrosyl peptides of defined sequences and chemical homogeneity permit straightforward colorimetric kinetic analysis of PTPases under conditions of full occupancy by the phosphorylated form of the substrate in the absence of large amounts of unphosphorylated substrate (as is the case in [^{32}P]phosphotyrosyl protein assays that monitor [^{32}P]P_i release). They will permit direct comparison of PTPases, for example, one-domain vs two-domain forms of the cytoplasmic fragment of LAR PTPase, LAR PTPase vs CD45 two-domain soluble fragments, full-length PTPases vs "core" catalytic fragments, and other members of the PTPase superfamily. The phosphotyrosyl peptides prepared in this study are all from the phosphotyrosyl sites in tyrosine kinases, but a variety of phosphotyrosyl sites in proteins that are not themselves protein tyrosine kinases are becoming known. Thus, $p34^{cdc2}$ (Draetta et al., 1988), phospholipase C1γ (Meisenhelder et al., 1989), and GAP (Ellis et al., 1990) are three proteins of consequence in signal transduction that undergo reversible phosphorylation/dephosphorylation on tyrosine for which catalytic parameters of the relevant PTPases have yet to be determined.

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Investigation of the Enzymatic Mechanism of Yeast Orotidine-5′-Monophosphate Decarboxylase Using ^{13}C Kinetic Isotope Effects†

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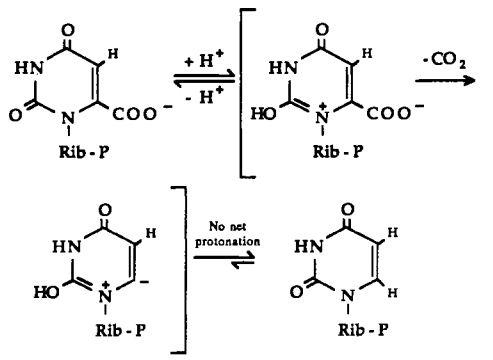
ABSTRACT: Orotidine-5′-monophosphate decarboxylase (ODCase) from *Saccharomyces cerevisiae* displays an observed ^{13}C kinetic isotope effect of 1.0247 ± 0.0008 at 25 °C, pH 6.8. The observed isotope effect is sensitive to changes in the reaction medium, such as pH, temperature, or glycerol content. The value of 1.0494 ± 0.0006 measured at pH 4.0, 25 °C, is not altered significantly by temperature or glycerol, and thus the intrinsic isotope effect for the reaction is apparently being observed under these conditions and decarboxylation is almost entirely rate-determining. These data require a catalytic mechanism with freely reversible binding and one in which a very limited contribution to the overall rate is made by chemical steps preceding decarboxylation; the zwitterion mechanism of Beak and Siegel [Beak, P. & Siegel, B. (1976) *J. Am. Chem. Soc. 98*, 3601-3606], which involves only protonation of the pyrimidine ring, is such a mechanism. With use of an intrinsic isotope effect of 1.05, a partitioning factor of less than unity is calculated for ODCase at pH 6.0, 25 °C. A quantitative kinetic analysis using this result excludes the possibility of an enzymatic mechanism involving covalent attachment of an enzyme nucleophile to C-5 of the pyrimidine ring. The observed isotope effect does not rise to the intrinsic value above pH 8.5; instead, the observed isotope effects at 25 °C plotted against pH yield an asymmetric curve that at high pH plateaus at about 1.035. These data, in conjunction with the pH profile of V_{max}/K_m, fit a kinetic model in which an enzyme proton necessary for catalysis is titrated at high pH, thus providing evidence for the catalytic mechanism of Beak and Siegel (1976).

Orotidine-5′-monophosphate decarboxylase (ODCase,[1] EC 4.1.1.23), catalyzes the conversion of OMP to UMP in the sixth and final step of the de novo pyrimidine biosynthetic pathway. The net reaction involves substitution of the carboxylate moiety at carbon 6 of the pyrimidine ring with a proton, and the reverse reaction has never been observed.

Scheme I: Decarboxylation of OMP by ODCase via Non-covalent, Zwitterion Mechanism[a]



[a] Intermediates in brackets exist at the enzyme active site.

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