Distinct functional roles of the two intracellular phosphatase like domains of the receptor-linked protein tyrosine phosphatases LCA and LAR

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Communicated by C. Weissman

Protein tyrosine phosphorylation is regulated by both protein tyrosine kinases and protein tyrosine phosphatases (PTPases). Recently, the structures of a family of PTPases have been described. In order to study the structure–function relationships of receptor-linked PTPases, we analyzed the effects of deletion and point mutations within the cytoplasmic region of the receptor-linked PTPases, LCA and LAR. We show that the first of the two domains has enzyme activity by itself, and that one cysteine residue in the first domain of both LCA and LAR is absolutely required for activity. The second PTPase like domains do not have detectable catalytic activity using a variety of substrates, but sequences within the second domains influence substrate specificity. The functional significance of a stretch of 10 highly conserved amino acid residues surrounding the critical cysteine residue located in the first domain of LAR was assessed. At most positions, any substitution severely reduced enzyme activity, while missense mutations at the other positions tested could be tolerated to varying degrees depending on the amino acid substitution. It is suggested that this stretch of amino acids may be part of the catalytic center of PTPases.

Key words: cassette mutagenesis/CD45/leukocyte common antigen/protein tyrosine phosphorylation

Introduction

Phosphorylation of proteins is a fundamental mechanism for regulating diverse cellular processes. Of particular interest in recent years has been the role of protein tyrosine phosphorylation in the signaling of cell growth, differentiation, proliferation and transformation. A large number of protein tyrosine kinases (PTKs) have been characterized (reviewed by Hunter, 1987), and the PTK activity of certain growth factor receptors and cellular oncogenes has been shown to be essential for their biological properties (Chou *et al.*, 1987; Kmiecik and Shalloway, 1987; Morgen and Roth, 1987). However, the level of protein tyrosine phosphorylation of intracellular substrates is determined by the balance of PTK and protein tyrosine phosphatase (PTPase) activities. Therefore, an understanding of the regulation of protein tyrosine phosphorylation requires knowledge of both PTK and PTPases.

While the biochemical properties of PTPases have been investigated (reviewed by Lau *et al.*, 1989), the primary

structures of a family of PTPases have been determined or deduced from cDNA cloning only recently. The amino acid sequence determination of the prototype member of this family, the placenta, cytosolic PTPase-1B (Charbonneau *et al.*, 1988), revealed that cell surface glycoproteins, the leukocyte common antigen (LCA, CD45) (Shen *et al.*, 1985; Thomas *et al.*, 1985; Ralph *et al.*, 1987; Streuli *et al.*, 1987) and LAR (Streuli *et al.*, 1988), contain in their cytoplasmic regions two domains that have ~ 25–40% amino acid sequence similarity to the PTPase-1B sequence. The exon–intron organization of the LCA gene suggests that these tandem domains arose from gene duplication (Hall *et al.*, 1988; Saga *et al.*, 1988; Johnson *et al.*, 1989). The structures of a second cytosolic PTPase TC-PTPase (Cool *et al.*, 1989), and two *Drosophila* PTPases, DLAR and DPTP (Streuli *et al.*, 1989) have also been predicted based on the sequences of cDNA clones. The PTPase activities of PTPase-1B, LCA, LAR, DLAR and DPTP have been examined using either purified PTPases (Tonks *et al.*, 1988a, 1988b), or recombinant PTPases derived from various expression systems (Ostergaard *et al.*, 1989; Streuli, *et al.*, 1989; Kiener and Mittler, 1989; Guan *et al.*, 1990).

The extracellular regions of LAR, DLAR and DPTP are structurally similar to the extracellular regions of a family of cell adhesion molecules that includes the neural cell adhesion molecule (N-CAM) (Streuli *et al.*, 1989). The LCA family is comprised of at least five different isoforms, which are generated by alternative splicing of three exons encoding extracellular amino-proximal sequences (Streuli *et al.*, 1987; Streuli and Saito, 1989). Thus, the structures of the LCA, LAR, DLAR and DPTP molecules suggest the possibility that the intracellular PTPase activity may be regulated by interactions between these extracellular domains and their ligands. LCA is expressed on hematopoietic cells, and LCA isoform expression is regulated in a lineage and developmentally specific manner (reviewed by Thomas, 1989). LCA is thought to play a role in lymphocyte activation (Rudd *et al.*, 1987; Ledbetter *et al.*, 1988; Pingel and Thomas, 1989; Ostergaard *et al.*, 1989; Schraven *et al.*, 1989). In contrast to LCA, LAR is expressed mainly on cells of non-hematopoietic origin (our unpublished results). The function of LAR is not yet known, but the overall structure of LAR (i.e., a cell-adhesion molecule like receptor linked to a cytoplasmic tyrosine phosphatase) suggests a possible role for LAR in the negative regulation of cell growth via cell-to-cell interactions. Supporting this hypothesis is the finding that the product of a putative colorectal tumor suppressor gene, DCC, is structurally similar to the extracellular region of LAR (Fearon *et al.*, 1990).

We previously demonstrated that one cysteine residue in the first of the two LCA PTPase domains is absolutely required for PTPase activity, and suggested that the second PTPase like domain of LCA may not have catalytic activity (Streuli *et al.*, 1989). This finding was unexpected because

all known receptor-linked PTPases have two PTPase-1B like domains, and all PTPase-1B like sequences have two cysteine residues in the same relative positions. Furthermore, the amino acid sequences of these second PTPase domains are relatively well conserved between species; in the case of human LAR versus *Drosophila* DLAR, the second PTPase domains are even better conserved than the first domains (83% versus 72% amino acid sequence similarity) (Streuli *et al.*, 1989). Here, we extend our initial mutational analysis of LCA PTPases to LAR, and demonstrate that although the second PTPase-1B like domains of LCA and LAR do not have enzymatic activity, they do affect the substrate specificity of the first PTPase domain. Furthermore, we show that a stretch of conserved amino acid residues surrounding the cysteine are critical for enzymatic activity, suggesting that these residues may be an important part of the PTPase catalytic site.

Results

Lack of catalytic activity in the second PTPase like domains of LCA and LAR

In order to explain the apparent lack of catalytic activity of the second PTPase-like domain of LCA, several possibilities have been considered. First, the second domain does have catalytic activity but with different substrate specificity than the first domain. Second, the second domain does have catalytic activity but that the activity is regulated. Third, the second domain is required for substrate binding and/or specificity but not catalytic activity (Streuli *et al.*, 1989). To address the functional significance of the second PTPase like domain, which is found in all of the receptor-linked PTPases, we initially determined the catalytic activity of the PTPases using various substrates including [³²P]Tyr-labeled peptides and [³²P]Tyr-labeled myelin basic protein (MBP). The cytoplasmic domains of LCA, LAR and mutants derived from these two PTPases were produced in *Escherichia coli* (Streuli *et al.*, 1989; see also Materials and methods). cDNA sequences encoding the cytoplasmic domains of LCA and LAR PTPases were inserted into the prokaryotic expression vector pKKUC-12. The recombinant plasmids were transformed into *E. coli* DHB4 (Δ *phoA*) cells, and bacterial extracts were prepared following IPTG induction. The level of PTPase activity in the extracts was determined by incubating an appropriate amount of the extracts with [³²P]Tyr-labeled substrates prepared as previously described, and measuring the amount of inorganic [³²P]phosphate released. As shown in Figure 1, the LCA PTPase has ~ 6-fold higher activity with the phosphotyrosyl-Raytide peptide substrate than it does with the phosphotyrosyl-MBP substrate. The LAR PTPase, in contrast, had relatively low activity with the phosphotyrosyl-Raytide, but a 9-fold higher activity with the phosphotyrosyl-MBP substrate (Figure 1). The 54-fold difference in the ratios of LAR and LCA PTPases activities with the two substrates suggests that the LCA and LAR PTPases have distinct substrate specificities.

To examine the contribution of the individual PTPase like domains of the LAR PTPase, a cysteine residue in each domain thought to be essential for enzyme activity was changed to a serine residue by site-directed mutagenesis as previously described for the LCA PTPase (Streuli *et al.*, 1989). The LCA PTPase with the Cys to Ser substitution

at amino acid position 828 (in this text, this mutation will be abbreviated as LCA C828S) had no detectable activity with either the phosphotyrosyl-Raytide (Streuli *et al.*, 1989) or the phosphotyrosyl-MBP substrate. The activity of the LCA C1144S (with the Cys to Ser mutation in the second LCA PTPase domain) was essentially wild type. Similar to LCA PTPase, the second conserved cysteine in the first LAR PTPase domain (LAR Cys1522) is also absolutely required for activity using either the phosphotyrosyl-Raytide peptide or the phosphotyrosyl-MBP substrate (Figure 1). The conserved cysteine residue in the second PTPase domain of LAR is not required for LAR enzyme activity. The wild type LCA and LAR PTPases can hydrolyze *p*-nitrophenylphosphate (pNPP). However, the LCA C828S and LAR C1522S mutant PTPases did not hydrolyze pNPP (data not shown). Therefore, no PTPase activity was demonstrated for the second PTPase like domain of LCA and LAR using a variety of substrates; this suggests that the lack of activity of the second PTPase like domains may not simply be a problem of substrate specificity.

Effects of the second PTPase like domain of LCA and LAR on the activity of the first domain

Although the second domain of the LCA and LAR PTPases had no detectable PTPase activity, we thought that the second PTPase like domain might have some influence on PTPase activity. To test this possibility, deletion mutants of LCA and LAR were created and expressed in *E. coli* (Figure 2A). Deletion of either the entire second domain of LCA [Δ (924–1281)] or an internal sequence therein [Δ (924–1109)] severely reduced, but did not completely destroy PTPase activity. These results demonstrate that the first LCA PTPase domain has detectable activity by itself and that the second PTPase domain might stabilize the first domain. A deletion mutation [Δ (828–1143)], which creates a hybrid PTPase domain by fusing the LCA Cys828 with the Cys1144, also had no detectable activity with either substrate. Therefore, the sequences 3' of LCA Cys828 cannot be substituted for by the amino acids in the second PTPase domain.

Following the second PTPase like domain of LCA there is a stretch of ~ 60 amino acids which is not homologous to any other PTPase but is conserved between the human and rodent LCAs. Because sequences at the carboxy terminus of the PTK p60^{c-src} have been shown to inhibit kinase activity (reviewed by Jove and Hanafusa, 1987), we tested whether or not the LCA carboxy-terminal sequence might influence PTPase activity. The deletion of the carboxy-terminal 47 amino acids from the LCA cytoplasmic region [Δ (1235–1281)] had no significant effect on LCA PTPase activity (Figure 2A). The carboxy-terminal sequence also did not act as an inhibitory sequence specific for the second LCA PTPase domain, because a double mutant PTPase in which the 3'-terminal deletion was combined with the Cys828 to Ser mutation [C828S, Δ (1275–1281)] did not have any enzyme activity. Another unusual feature of the second LCA cytoplasmic domain is that there is a 19 amino acid insertion which is rich in serine and acidic amino acids. This insertion is conserved between human, murine and rat LCA cytoplasmic domains, but is not found in any of the other PTPases. Removal of these 19 amino acids [Δ (969–987)] from the LCA PTPase resulted in a 6.5-fold increase in activity with phosphotyrosyl-MBP, but did not

Fig. 1. Schematic diagrams of the structures of the cytoplasmic domains of LCA, LAR, and of cytoplasmic domain mutants containing cysteine to serine missense mutations expressed in *E. coli*, and their level of PTPase activities. cDNA sequences encoding the LCA and LAR cytoplasmic domains, without or with missense mutations, were inserted into the prokaryotic expression vector pKKUC-12, and extracts of DHB4 cells harboring these hybrid plasmids were prepared as described in Materials and methods. The LCA cDNA and LAR cDNA segments inserted into the expression vector encode amino acid residues 584–1281, and 1275–1881, respectively (amino acid numbers are from Streuli *et al.*, 1987, and 1988, respectively). Also indicated are the amino acid positions of conserved cysteine (C) and cysteine to serine (S) missense mutations. The amount of PTPase activity in the extracts using either phosphotyrosyl-Raytide or phosphotyrosyl-myelin basic protein (MBP) substrate was determined as described in Materials and methods. Boxes indicate the relative size and positions of the first (Cy-1) and second (Cy-2) PTPase-1B like domains in the LCA (open boxes) and LAR (closed boxes) cytoplasmic regions.

cause any significant change in activity with phosphotyrosyl-MBP, but did not cause any significant change in activity with phosphotyrosyl-Raytide (Figure 2A). Thus, the ratio of PTPase activity with the phosphotyrosyl-MBP to -Raytide substrates for the wild type LCA and the LCA $\Delta(969-987)$ was altered ~ 7.5 -fold. To test whether the increased activity with the MBP substrate resulted from catalytic activation of the second LCA PTPase domain, a double mutation was constructed that combined the $\Delta(969-987)$ deletion with the C828S missense mutation. The double mutant had no detectable enzyme activity (Figure 2A). Therefore, the effect of $\Delta(969-987)$ was to alter the specificity of the first domain, rather than to activate the latent catalytic activity of the second domain. Taken together, the deletion analysis suggests that the second LCA PTPase domain may have a regulatory role in controlling the substrate specificity of the catalytically active first domain.

To establish if the second domain of LAR also has a regulatory role, we tested the effects of deletions in the cytoplasmic region of LAR on PTPase activity (Figure 2B). Deletion of about half of the second PTPase like domain [$\Delta(1716-1881)$] did not significantly affect the activity of the LAR PTPase with either the phosphotyrosyl-Raytide or the phosphotyrosyl-MBP substrate. However, removal of the entire second PTPase domain [$\Delta(1614-1881)$] resulted in ~ 2 -fold increase in activity with the Raytide substrate, and an 8.5-fold decrease in activity with the MBP substrate. The ratios of activity with the phosphotyrosyl-MBP to -Raytide for the wild type LAR and LAR $\Delta(1614-1881)$ was altered

~ 20 -fold. This result indicates that the second LAR PTPase domain also affects substrate specificity, consistent with the finding for the second LCA PTPase domain. Additional sequence requirements of the LAR PTPase were investigated by testing the effects of amino- and carboxy-terminal deletions on PTPase activity. Deletion of amino-terminal [$\Delta(1275-1342)$] or [$\Delta(1275-1311)$] or carboxy-terminal amino acids [$\Delta(1551-1881)$] respectively from the first domain of the LAR PTPase resulted in complete loss of enzyme activity. A LAR deletion mutation [$\Delta(1522-1812)$] which creates a hybrid PTPase domain by fusing the LAR Cys1522 with the Cys1813 residue had no activity. Therefore, sequences from the second half of the first LAR domain (amino acids 1523–1613) cannot be replaced functionally by sequences (1814–1881) from the second half of the second LAR domain even though these sequences are homologous. Deletion of most of the first LAR PTPase domain [$\Delta(1275-1529)$] also completely destroyed enzyme activity. Therefore, based on the deletion analysis of the LAR cytoplasmic domain, the minimum sequence requirement for LAR PTPase activity is from amino acid positions 1275 to 1613. Deletions of amino acid positions 1614–1715 from the second domain affect the substrate specificity of the first domain. Thus, for both LCA and LAR, the second PTPase like domains seem to be important for substrate specificity. However, the possibility that the second PTPase domains have intrinsic PTPase activity with suitable substrates, or that the enzyme activity requires an activation signal such as phosphorylation, cannot be completely excluded.

Fig. 2. Analysis of the effect of deletion mutations in the cytoplasmic regions of (A) LCA, and (B) LAR on PTPase activity. Symbols and determination of PTPase activities are as described in Figure 1 legend.

Mutational analysis of highly conserved amino acid residues in LAR PTPase

Alignment of 10 known PTPase like sequences (PTP-1B, TCPTP and two each for LAR, LCA, DLAR and DPTP) reveals that ~ 10% of the amino acid positions are absolutely conserved (Streuli *et al.*, 1989). The number of

absolutely conserved amino acid positions is higher (nearly 15%) if only the first domains of the receptor-type PTPases are included in the comparison. Furthermore, almost all of these positions are invariant when five newly identified human PTPase sequences (NXK and HS, unpublished data) are included in the comparison. These conserved positions,

LAR	Cy1	(1516-1530)	G	P	M	V	V	H	C	S	A	G	V	G	R	T	G
LCA	Cy1	(822-836)	-	-	I	-	-	-	-	-	-	-	-	-	-	-	-
DLAR	Cy1	(1632-1646)	-	-	V	I	-	-	-	-	-	-	-	-	-	-	-
DPTP	Cy1	(1068-1082)	-	-	I	L	-	-	-	-	-	-	-	-	-	-	-
PTP 1B		(209-223)	-	-	V	-	-	-	-	-	I	-	-	-	-	S	-
TCPTP		(210-224)	-	-	A	I	-	-	-	-	I	-	-	-	S	-	-
LAR	Cy2	(1807-1821)	-	-	I	T	-	-	-	-	-	-	-	-	-	-	-
LCA	Cy2	(1138-1152)	T	-	L	L	I	-	-	R	D	-	S	Q	Q	-	-
DLAR	Cy2	(1823-1837)	-	-	I	T	-	-	-	-	-	-	-	-	-	S	-
DPTP	Cy2	(1362-1376)	S	-	L	T	-	-	-	L	T	D	-	S	S	-	-

Fig. 3. Alignment of a stretch of highly conserved amino acid residues found in all members of the PTPase family. Amino acid sequences from the first (Cy-1) and second (Cy-2) PTPase like cytoplasmic domains of LAR (Streuli *et al.*, 1988), LCA (Streuli *et al.*, 1987), DLAR (Streuli *et al.*, 1989), DPTP (Streuli *et al.*, 1989), as well as, the cytosolic PTPases, PTP-1B (Charbonneau *et al.*, 1989) and TC-PTP (Cool *et al.*, 1989), are compared with the LAR sequence. Numbers indicate positions of the amino acid residues compared. The standard one letter amino acid code is used. Dashes (—) indicate identical amino acid residues as compared with the LAR Cy-1 sequence.

therefore, are probably important either for the enzyme catalytic reactions *per se* or for the proper folding of the enzyme domains. Particularly striking among these conserved amino acid positions is the stretch of 15 amino acids around the conserved LAR Cys1522 residue (Figure 3). Within this stretch, 10 positions are invariant among the cytosolic PTPases and the first domains of the receptor-type PTPases, and the remaining five positions have only conserved substitutions (such as Val to Ile, or Thr to Ser). On the other hand, the sequences of the second domains show much greater variation with frequent non-conservative substitutions, such as Val to Thr, or Ala to Asp. In addition to the high level of conservation, this stretch of amino acids has another feature that attracts attention. PTPases are known to require a reduced cysteine residue for activity (Tonks *et al.*, 1988a). Because there are only two conserved cysteines in each PTPase domain, and one of the cysteines is dispensable (Streuli *et al.*, 1989), the cysteine at position 1522 of LAR is very likely to be at the catalytic center. In order to determine the importance of these amino acid positions for PTPase activity and/or specificity, we systematically altered several of these residues (i.e., V1520, H1521, S1523, A1524, G1525, V1526 and R1528) to a number of different amino acid residues. To generate mutants we used a variation of the cassette mutagenesis technique (Wells *et al.*, 1985). A pair of complementary oligonucleotides were synthesized that contained a mixture of all four bases at the first and second positions of one codon within the cassette. Using this approach, a codon of interest can be mutated to a large number of different codons. Testing the effect of several missense mutations at any one amino acid residue is important because the functional significance of an amino acid residue may not be unmasked by only one type of missense mutation. The oligonucleotide cassette was inserted between two restriction sites flanking the segment of DNA to be mutated, and resulting plasmids containing missense mutations were screened by sequencing (see Figure 4). Restriction fragments encoding amino acid substitutions were then used to replace sequences in the pKKUC-LAR Δ (1614–1881) construct (Figure 2B; abbreviated as pKKUC-LAR D1). The one domain LAR construct pKKUC-LAR D1 was chosen, as opposed to the wild type pKKUC-LAR, in order to simplify the interpretation of the effect of amino acid substitutions on enzyme activity. The

pKKUC-LAR D1

Fig. 4. Cassette mutagenesis of the LAR PTPase. A schematic diagram of the pKKUC-LAR D1 construct is shown, with an enlargement below showing the nucleotide and amino acid (one letter code) sequence that was mutagenized. The pKKUC-LAR D1 construct is the same as the pKKUC-LAR Δ (1614–1881) construct (Figure 2B), and encodes only the first LAR PTPase domain. The open box represents the LAR cDNA segment (encoding amino acids 1275–1613), with restriction sites shown above; the shaded box indicates the prokaryotic tcr promoter (P_{tcr}) region of the pKKUC-12 vector. A thin line represents other vector sequences. Oligonucleotide cassettes containing a mixture of all four bases at the first and second positions of the codon to be mutated were inserted between the *ApaI* and *BglI* sites, or between the *PstI* and *BglI* sites.

LAR D1 P1517L and C1522S mutations were generated by oligo-directed mutagenesis as described previously (Foss and McClain, 1987). pKKUC-LAR D1 plasmids containing the various codon mutations were transformed into *E. coli* DHB4 cells, extracts were prepared from bacterial cultures, and the level of PTPase activity was determined for each mutant LAR using both the phosphotyrosyl-Raytide and phosphotyrosyl-MBP substrates. In general, the percentage change in the level of activities of the mutant LAR PTPases using either substrate was not significantly different from one another (Figure 5).

Essentially any substitutions at several LAR amino acid positions (i.e. Cys1522, Ala1524, Gly1525 and Arg1528) caused loss of enzyme activity (Figure 5). Amino acid substitutions at other amino acid positions such as Pro1517, Val1520, His1521, Ser1523 and Val1526 were tolerated to varying degrees depending on the nature of the substitution (Figure 5). The LAR Pro1517 to Leu mutant had ~ 1.5% of wild type (wt) LAR D1 PTPase activity. LAR D1 Val1520 mutations fell roughly into three groups; Val to Met or Leu substitutions had 8–45% of wt activity; Val to Gly or Thr had only ~ 0.4% of wt activity, and Val to Glu, Gln, or Arg substitutions had no detectable activity. LAR D1 His1521 mutations also fell into roughly three groups: His to Asn, Cys, Gln, and Leu had 10–25% of wt activity; His to Ser or Thr substitutions had ~ 1–5% of wt activity; His to Ile, Phe, Asp, Tyr, Arg, or Ala substitutions had undetectable to ~ 0.5% of wild type activity. Substitution of Cys1522 to Ser had no detectable activity, consistent with the finding that this mutation in the full-length LAR also did not have any detectable activity. Ser1523 to Thr, Val, Asn, Cys, Tyr, His, Phe, or Ile substitutions all had very low but detectable (0.3–1.4%) activity with at least the phosphotyrosyl-Raytide substrate. Of all the LAR Ser1523 mutants tested, only the proline mutant had no detectable activity. LAR Ala1524 to Gly or Ser substitutions had ~ 12.6% or 0.4% of wt activity respectively, whereas other mutations at this position had no detectable activity. LAR Gly1525 to Ala or Ser mutant PTPases had ~ 0.4% of wt activity using the phosphotyrosyl-Raytide substrate, but other

mutations at Gly1525 had no detectable activity. LAR Val1526 substitutions fell into roughly four groups: Val to Ser or Leu substitutions had ~ 14% of wt activity; Val to Gln, Lys, Gly, Arg, or Thr had ~ 2%–10% of wt activity; Val to Trp or Glu had ~0.5% of wt activity; and the Val to Pro substitution had no detectable activity. Substitution of Arg1528 with any amino acid residue tested all had no detectable PTPase activity.

		PTPase activity units/mg extract	
		Raytide	MBP
Wild type	G P M V V H C S A G V G R T	844.7	394.8
P1517L	- L - - - - - - - - - -	10.6	6.9
V1520L	- - - - L - - - - - - - -	230.6	171.5
V1520M	- - - - M - - - - - - - -	134.0	32.3
V1520G	- - - - G - - - - - - - -	4.1	1.3
V1520T	- - - - T - - - - - - - -	3.8	1.7
V1520E	- - - - E - - - - - - - -	-	-
V1520Q	- - - - Q - - - - - - - -	-	-
V1520R	- - - - R - - - - - - - -	-	-
H1521H	- - - - - N - - - - - - - -	900.7	572.0
H1521N	- - - - - C - - - - - - - -	105.2	39.4
H1521C	- - - - - N - - - - - - - -	100.7	69.6
H1521Q	- - - - - Q - - - - - - - -	91.5	92.0
H1521L	- - - - - L - - - - - - - -	79.3	38.9
H1521T	- - - - - T - - - - - - - -	41.0	20.6
H1521S	- - - - - S - - - - - - - -	26.2	4.2
H1521I	- - - - - I - - - - - - - -	8.5	1.8
H1521F	- - - - - F - - - - - - - -	2.3	-
H1521D	- - - - - D - - - - - - - -	1.9	-
H1521Y	- - - - - Y - - - - - - - -	1.9	-
H1521R	- - - - - R - - - - - - - -	1.1	-
H1521A	- - - - - A - - - - - - - -	0.9	-
C1522S	- - - - - S - - - - - - - -	-	-
S1523S	- - - - - - - - - - - - - -	1114.0	651.4
S1523T	- - - - - T - - - - - - - -	11.7	0.9
S1523V	- - - - - V - - - - - - - -	6.7	1.1
S1523N	- - - - - N - - - - - - - -	5.5	1.6
S1523C	- - - - - C - - - - - - - -	3.7	-
S1523Y	- - - - - Y - - - - - - - -	3.2	-
S1523H	- - - - - H - - - - - - - -	3.1	-
S1523F	- - - - - F - - - - - - - -	2.6	-
S1523I	- - - - - I - - - - - - - -	2.6	-
S1523P	- - - - - P - - - - - - - -	-	-
A1524A	- - - - - - - - - - - - - -	838.0	456.0
A1524G	- - - - - G - - - - - - - -	108.1	49.1
A1524S	- - - - - S - - - - - - - -	3.5	1.6
A1524D	- - - - - D - - - - - - - -	-	-
A1524H	- - - - - H - - - - - - - -	-	-
A1524I	- - - - - I - - - - - - - -	-	-
A1524L	- - - - - L - - - - - - - -	-	-
A1524N	- - - - - N - - - - - - - -	-	-
A1524P	- - - - - P - - - - - - - -	-	-
A1524R	- - - - - R - - - - - - - -	-	-
A1524V	- - - - - V - - - - - - - -	-	-
A1524Y	- - - - - Y - - - - - - - -	-	-
G1525A	- - - - - A - - - - - - - -	3.3	-
G1525S	- - - - - S - - - - - - - -	2.8	-
G1525C	- - - - - C - - - - - - - -	-	-
G1525D	- - - - - D - - - - - - - -	-	-
G1525F	- - - - - F - - - - - - - -	-	-
G1525I	- - - - - I - - - - - - - -	-	-
G1525L	- - - - - L - - - - - - - -	-	-
G1525N	- - - - - N - - - - - - - -	-	-
G1525P	- - - - - P - - - - - - - -	-	-
G1525R	- - - - - R - - - - - - - -	-	-
G1525T	- - - - - T - - - - - - - -	-	-
G1525V	- - - - - V - - - - - - - -	-	-
G1525Y	- - - - - Y - - - - - - - -	-	-
V1526S	- - - - - S - - - - - - - -	152.9	51.3
V1526L	- - - - - L - - - - - - - -	121.9	41.7
V1526Q	- - - - - Q - - - - - - - -	80.6	33.7
V1526K	- - - - - K - - - - - - - -	56.8	5.9
V1526G	- - - - - G - - - - - - - -	32.9	10.2
V1526R	- - - - - R - - - - - - - -	31.1	12.2
V1526T	- - - - - T - - - - - - - -	20.0	8.6
V1526W	- - - - - W - - - - - - - -	7.2	1.5
V1526E	- - - - - E - - - - - - - -	3.5	-
V1526P	- - - - - P - - - - - - - -	-	-
R1528R	- - - - - - - - - - - - - -	929.7	605.7
R1528C	- - - - - C - - - - - - - -	-	-
R1528D	- - - - - D - - - - - - - -	-	-
R1528G	- - - - - G - - - - - - - -	-	-
R1528I	- - - - - I - - - - - - - -	-	-
R1528K	- - - - - K - - - - - - - -	-	-
R1528L	- - - - - L - - - - - - - -	-	-
R1528P	- - - - - P - - - - - - - -	-	-
R1528S	- - - - - S - - - - - - - -	-	-
R1528T	- - - - - T - - - - - - - -	-	-
R1528V	- - - - - V - - - - - - - -	-	-

Discussion

We have examined the structure–function relationships of PTPases by testing in an *in vitro* assay system the effects of deletion or missense mutations in the cytoplasmic domains of the LCA and LAR PTPases. The finding that a cysteine to serine missense mutation in the first of the two homologous PTPase domains of both LCA and LAR (LCA C828S, LAR C1522S) destroyed enzyme activity demonstrated that this residue is absolutely required for catalytic activity, and at the same time suggested that the second of the two repeat PTPase-1B like domains of LCA and LAR did not have intrinsic catalytic activity. Although the possibility that the second domains do have catalytic activities that were not detected in our assay system remains, we believe that the second domains do not have intrinsic catalytic activity for several reasons. First, hydrolysis of three widely different substrates, phosphotyrosyl-protein, phosphotyrosyl-oligopeptide and *p*-nitrophenylphosphate depends on the activity of the first domain. Neither the LCA C828S nor the LAR C1522S PTPases are active with any of these substrates, and it is very unlikely that a Cys to Ser mutation in the first PTPase domains could induce a conformational change in the second domain resulting in its inactivation. Second, the sequence around LCA Cys1144 (TPLLIHCRDGSQQTG) contains several amino acid residues that severely reduce phosphatase activity when introduced into the analogous positions of the first domain of LAR (Figures 3 and 5). For example, the aspartic acid residue at position 1146 of LCA might be one of the reasons that the second domain of LCA is inactive, because in LAR the Ala 1524 to Asp mutation completely inactivated LAR activity. Several amino acid positions around the cysteine of the second domain of DPTP would also be expected to reduce the catalytic activity. For example, the leucine residue at position 1370 (instead of alanine) would completely abolish activity. Further support for the idea that the second domains of the receptor-linked phosphatase may not, or need not, be catalytically active comes from the structural analysis of newly isolated receptor-linked PTPases. A phosphatase was found that has two PTPase like domains with a significant level of similarity to any of the other PTPases, but in which the second domain does not have a conserved cysteine which is thought to be essential for catalytic activity (N.X.Krueger and H.Saito, unpublished results).

Fig. 5. Activities of LAR PTPases containing missense mutations at highly conserved amino acid residues. At the top of the figure is shown the amino acid sequence (one letter code) of the region located in the first LAR PTPase domain that was mutated by cassette mutagenesis, and the PTPase activities of the pKKUC-LAR D1-derived PTPase (see Figure 4). PTPase activities using the phosphotyrosyl-Raytide and phosphotyrosyl-myelin basic protein (MBP) substrates were determined as described in Materials and methods. Below the sequence and PTPase activities of the pKKUC-LAR D1-derived PTPase, which is defined here as 'wild type', are the sequences of the missense mutants, and their PTPase activities. Mutant LAR PTPases are designated so as to indicate the wild type residue mutated, the amino acid position, and the missense residue. For example, P1517L, indicates that the pKKUC-LAR D1-derived PTPase has the proline (P) at position 1517 mutated to a leucine (L) residue. H1512H, S1523S, A1524A and R1528R PTPases are essentially the same as wild type, except that they were derived via the mutagenesis procedure. Dashes (–) below the amino acid sequence indicate identical residues as in the wild type sequence. Dashes (–) in right hand side of the Figure indicate that the mutant PTPase had undetectable levels of activity.

What is the functional role of the second PTPase like domain of LCA and LAR if it is not catalytic? The finding that certain deletion mutations within the second domain of LCA and LAR can either increase or decrease the activity of LCA or LAR towards the phosphotyrosyl-MBP, without diminishing the activity towards the phosphotyrosyl-Raytide substrate suggests that the second domain may regulate substrate specificity or receptor-linked PTPases. Possibly, interactions with the short peptide phosphotyrosyl-Raytide requires only sequences in the first domain, while binding of the phosphotyrosyl-MBP involves sequences in both domains. This interpretation is best supported by the finding that the 54-fold difference in the ratios of activities of LCA and LAR towards the phosphotyrosyl-Raytide and phosphotyrosyl-MBP substrates can be altered to only a 3-fold difference by manipulating the second domain of LCA [$\Delta(969-987)$] and LAR [$\Delta(1614-1881)$]. Furthermore, if the second domain of the receptor-linked PTPases has a regulatory function, then the regulation of the one domain cytosolic PTPases must be different from the receptor-type PTPases.

Mutational analysis of several highly conserved amino acid positions around the critical LAR Cys1522 residue suggests that this region may be directly involved in the catalytic process. Missense mutations of LAR amino acid residues Pro1517, Cys1522, Ser1523, Ala1524, Gly1525 and Arg1528, even with very conservative residues (except for the Ala1524 to Gly mutation), either abolished, or severely reduced activity to < 2% of wild type LAR D1 PTPase activity. The finding that essentially all mutations generated within the conserved segment reduced PTPase activity is significant because, in general, a majority of amino acid substitutions are neutral mutations in many other proteins. Moreover, ~ 90% of hydroxylamine-induced mutations, which are located throughout the first LAR PTPase domain, had no detectable effect on enzyme activity (A. Tsai and H. Saito, unpublished results). Therefore, the amino acid residues in the conserved LAR segment must be essential for function and/or structure, and are possibly part of the active site of the enzyme. Given the importance of these amino acid residues for PTPase activity, how might they be involved in the mechanistic pathway of phosphotyrosyl protein dephosphorylation? *A priori*, there are two general categories of phosphatase mechanisms: one-step hydrolysis with water as the nucleophilic acceptor and two-step hydrolysis with a phosphoenzyme intermediate. Whereas both mechanisms are theoretically possible, the catalytic mechanism of many hydrolases, especially enzymes involved in phosphoryl transfer reactions (reviewed by Knowles, 1980), suggests that a mechanism involving a phosphoenzyme intermediate might be more likely for PTPases.

With the serine protease paradigm, chymotrypsin, a nucleophilic amino acid at the catalytic center initiates the hydrolytic reaction by attacking an electrophilic group of the substrate. For serine proteases, the nucleophile is a serine hydroxyl group partially deprotonated by a charge relay system composed of a histidine and an aspartic acid residue (reviewed by Walsh, 1979). Alkaline phosphatases also use a serine residue as the enzyme nucleophile. However, the Ser1523 of LAR cannot be the catalytic nucleophile of the LAR PTPase, because substitutions of this serine with many other amino acids did not completely abolish the catalytic activity. Furthermore, PTPases do not have any other

conserved serine residues, suggesting that serine is not the PTPase nucleophile. Other common nucleophiles found among hydrolases include cysteine and histidine residues, which are in fact both found within the highly conserved sequence of PTPases. Histidine at position 1521 of LAR cannot however be the nucleophile because many substitutions at this position can be tolerated to some extent. Furthermore, the conserved LAR His1521 residue probably is not involved in a charge relay system because a PTPase containing a His1521 to Leu missense substitution still had ~ 14% of wild type activity. It is unlikely that a charge relay system would be functional after substitution with the non-polar amino acid residue leucine for the charged amino acid residue histidine. We previously demonstrated that the first conserved cysteine residue in the first domain of LCA (C737) is dispensable (Streuli *et al.*, 1989), suggesting that the analogous conserved cysteine residue in LAR (C1434) is also dispensable. Thus, the cysteine at position 1522 remains as a possible nucleophile. The conserved cysteine residue in the first domain of LAR (C1522) as well as LCA (C828) cannot be substituted for, even with the very conservative serine residue, without completely abolishing enzyme activity. Enzymes that use cysteine as a catalytic nucleophile have been described, including cysteine proteases (reviewed by Walsh, 1979). Although nucleophilic attack of electrophilic phosphorus by a cysteine has not been observed among characterized enzymes, phosphocysteinyl linkages have been detected in thioredoxin (Conely and Pigiet, 1978). Because other potentially nucleophilic amino acids such as aspartic acid, glutamic acid and threonine are also among the conserved amino acid residues of PTPases (Streuli *et al.*, 1989), it is premature to assign the cysteine residue at LAR position 1522 to the catalytic center. Nevertheless, the highly conserved sequence surrounding the cysteine distinguishes this amino acid from all other potential nucleophiles. Obviously, the previous observation that PTPase activity is sensitive to cysteinyl-modifying agents (Tonks *et al.*, 1988a) supports the possibility that a cysteine residue is an important part of the catalytic center.

The functional significance of the LAR Arg1528 residue, which is absolutely required for enzyme activity, may be to neutralize the negative charge of the phosphoryl group in a fashion similar to the Zn^{2+} cation of alkaline phosphatases. In fact, LAR does not require any metal cations. The LAR amino acid residues Val1520, Ala1524, Gly1525 and Val1526, which all have non-polar side chains unlikely to be directly involved in dephosphorylation, might be important for maintaining local enzyme structure. Often the catalytic center of enzymes is buried within a very hydrophobic environment. However, establishing the definite role of the cysteine residue and other critical amino acid residue in the PTPase enzyme mechanism will require additional mutational and kinetic analyses, demonstration of a phosphoenzyme intermediate, and ultimately a three dimensional structure of the enzyme.

Materials and methods

Buffers

Lysozyme buffer: 33 mM Tris-HCl, pH 8.0/2.5 mM EDTA/10 mM 2-mercaptoethanol. 10 × phosphatase buffer: 250 mM HEPES, pH 7.3/50 mM EDTA/100 mM dithiothreitol.

Substrates for phosphatase assays

The synthetic peptide Raytide (Oncogene Science, Manhasset, NY) or bovine brain myelin basic protein (Sigma) was labeled at its tyrosine residue using [γ - 32 P]ATP and *v-abl* tyrosine kinase (Oncogene Science) essentially as described by the suppliers except that the reactions were incubated at 37°C for 12–16 h, and terminated by the addition of 0.5 ml of 20% (w/v) trichloroacetic acid (TCA)/20 mM NaH₂PO₄ and 0.1 ml 5 mg/ml BSA (Life Technologies, Inc). Following centrifugation, protein precipitates were washed three times with 20% (w/v) TCA/20 mM NaH₂PO₄, and then dissolved in 0.2 M Tris-HCl pH 8.0.

Bacterial extracts

Isopropyl β -D-thiogalactoside (final concentration, 1 mM) was added to exponentially growing cultures of *E. coli* DHB4 (Δ *phoA phoR* (F' *lacI^Q pro*)) (Boyd et al., 1987) carrying appropriate pKKUC plasmids, and cultures were shaken at 30°C for 2 h. Cells in 1.5 ml cultures were collected in a microfuge and resuspended in 0.2 ml of lysozyme buffer containing hen egg white lysozyme (1.0 mg/ml). After 10 min at room temperature, cells were lysed by three cycles of freezing–thawing. Ten microliters of 0.2 M MgCl₂ and 5 μ l of DNase I (5 mg/ml) were added to the lysates and incubated at 25°C for 20 min, followed by the addition of 20 μ l of 0.2 M EDTA and 26 μ l of 10% Triton X-100, and incubated at 25°C for 10 min. Protein contents of the extracts were determined according to the method of Bradford (1976). Extracts (40 μ l) were analyzed by SDS–PAGE, and gels were stained with Coomassie blue as described (Ausubel et al., 1989). All of the extracts contained distinguishable proteins of the expected mol. wt for the various mutant PTPases except the: pKKUC-LCA Δ (924–1281); -LCA Δ (924–1109); -LCA Δ (969–987); -LCA C828S, Δ (969–987); -LAR Δ (1275–1342), Δ (1614–1881); -LAR Δ (1275–1311), Δ (1614–1881); -LAR Δ (1552–1881); -LAR D1 P1517L; -LAR D1 V1250R; and -LAR D1 H1521D PTPases. The inability to observe certain mutant PTPases may be caused either because the expected protein comigrated with *E. coli* protein(s) or that the protein is degraded.

Phosphatase assay

The phosphatase assay mixture (50 μ l) contains 5 μ l of 10 $\times$ phosphate buffer, 5 μ l of radioactive substrate [32 P]Tyr-Raytide, or [32 P]Tyr-myelin basic protein (1–5 $\times$ 10⁵ c.p.m.) and 5 μ l of sample to be assayed. After incubation at 37°C for 30 min, the reactions were terminated by the addition of 0.75 ml of acidic charcoal mixture (0.9 M HCl/90 mM sodium pyrophosphate/2 mM NaH₂PO₄/4% (v/v) Norit A). After centrifugation in a microfuge, the amount of radioactivity in 0.4 ml of supernatant was measured. Enzyme activity was expressed in arbitrary units where 1 unit is the amount of protein phosphatase needed to release 3.33% of the total phosphate per min (Streuli et al., 1989).

Plasmid constructions

Construction of the prokaryotic expression plasmids, pKKUC-LCA and pKKUC-LAR, as well as the pKKUC-LCA C828S and pKKUC-LCA C1144S plasmids has been described previously (Streuli et al., 1989). Mutants of pKKUC-LCA and pKKUC-LAR plasmids containing cysteine to serine missense mutations, and the pKKUC-LCA Δ (969–987), pKKUC-LAR Δ (1275–1342), Δ (1614–1881), and pKKUC-LAR Δ (1552–1881) deletion mutations were constructed using *in vitro* site-directed mutagenesis essentially according to Foss and McClain (1987). The remaining pKKUC-LCA and -LAR deletion mutants were constructed by removing appropriate restriction fragments from the LCA and LAR cDNAs. The positions of the amino acid substitutions and deletions of the various constructions are given in Figures 1 and 2. The pKKUC-LAR Δ (1614–1881) construct encodes an additional 16 amino acids at the 3' end which are derived from vector sequence but do not affect enzyme activity (data not shown).

Cassette mutagenesis

Cassette mutagenesis was done essentially as described previously (Wells et al., 1985). Briefly, pairs of oligonucleotides corresponding to both strands of the LAR cDNA encoding amino acid positions 1516–1530 (*Apal*–*Bgl*I fragment) or 1522–1530 (*Pst*I–*Bgl*I fragment) were synthesized that contained equal amounts of the four nucleotides at the first and second positions of one of the codons in the cassette. The oligonucleotide cassette mixture was ligated to restriction fragments containing appropriate restriction sites (either LAR *Apal*–*Bgl*I, or *Pst*I–*Bgl*I, see Figure 4) of the plasmid pSP6-LAR D1, and *E. coli* DH5 cells were transformed with the ligation mixture. Some of the missense mutations were generated using non-degenerate oligonucleotide cassettes, while pKKUC-LAR D1 P1517L, H1521Q and C1522S mutants were generated by site-directed mutagenesis (Foss and McClain, 1987). Before sequence analysis of resulting plasmids, each plasmid was re-transformed in order to ensure the clonality of the mutants. The plasmid pSP6-LAR D1 was used as an intermediary vector,

instead of directly using the pKKUC-LAR Δ (1614–1881) (abbreviated as pKKUC-LAR D1) plasmid for the cassette mutagenesis, because the higher plasmid DNA yields of the pSP6-LAR D1 plasmids facilitated the sequencing analysis of mutants. Sequencing of plasmid DNA was done according to the method of Sanger et al. (1977) using genetically modified T7 DNA polymerase (Tabor and Richardson, 1989). Restriction fragments from the pSP6-LAR D1 plasmid (*Xho*I–*Bgl*I or *Xho*I–*Sph*I, see Figure 4), containing missense mutations, were then ligated to appropriate restriction fragments derived from the plasmid pKKUC-LAR D1 in order to generate pKKUC-LAR D1 plasmids containing missense mutations.

Acknowledgements

We thank Drs P. Anderson, M. Robertson and C. Walsh for critical reading of the manuscript; Drs C.C. Richardson and S. Tabor for sequence; and Dr S.F. Schlossman for his continual encouragement and support. M.S. is supported by a Cancer Research Institute Fellowship and H.S. is supported by an Investigator Award from the Cancer Research Institute/Jesselson Foundation Fellowship, a grant from American Cancer Society-Massachusetts Division (R-2-90), and grants from National Institutes of Health (SO7 RR-05526 and AI-26589).

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Received on April 9, 1990