The EMBO Journal vol.10 no.11 pp.3231 - 3237, 1991

The receptor-like protein tyrosine phosphatase HPTPα has two active catalytic domains with distinct substrate specificities

Yue Wang and Catherine J.Pallen

Cell Regulation Laboratory, Institute of Molecular and Cell Biology, National University of Singapore, 10 Kent Ridge Crescent, Singapore 0511, Republic of Singapore

Communicated by S.Brenner

Cloning and expression of the homologous domains of the receptor-like tyrosine phosphatase HPTPα shows that both domain 1 (D1) and domain 2 (D2) are enzymatically active. The two domains display different substrate specificities with D1 preferentially dephosphorylating MBP ~ RR-src > PNPP while D2 favours PNPP ⋙ RR-src and is inactive towards MBP. Each domain has lower activity than an expressed protein containing both domains. Analysis of chimaeric D1/2 proteins suggests that no particular region of D2 is responsible for the low activity of D2 on RR-src and that the specificity differences of D1 and D2 reflect overall sequence dissimilarities. Activities of D1 and D2 are inhibited by zinc, vanadate and EDTA and differentially susceptible to inhibition by heparin and poly(Glu4:Tyr1). Unusually, the activity of the protein containing both domains is stimulated by these polyanions. Regions amino-terminal to each domain are important for catalysis since deletion of these sequences abolishes phosphatase activity. Activity of the double domain polypeptide was also lost upon deletion of the sequence amino-terminal to D1, indicating that inactivation of D1 may suppress D2 activity. Differences in substrate specificity and responses to effectors and the interdependence between the two domains are likely important properties in the function of this PTPase in signal transduction.

Key words: homologous domains/PTPase/tandem active sites/tyrosine dephosphorylation

Introduction

Tyrosine phosphorylation is a common feature of signalling pathways which mediate cell proliferation and neoplastic transformation. The activation of growth factor receptor and non-receptor kinases initiates many of these events while tyrosine phosphatases (PTPases) were thought to play a crucial but more passive role in signal termination and in maintaining basal phosphotyrosine levels in the resting cell. Two major discoveries suggest that tyrosine phosphatases can also actively initiate unique reaction cascades. Firstly, the activity of a key cell cycle regulatory element, the cdc2 kinase, is switched on by tyrosine dephosphorylation (Dunphy *et al.*, 1989; Morla *et al.*, 1989; Gould *et al.*, 1989). Secondly, protein microsequencing of a purified placental tyrosine phosphatase, PTP 1B (Tonks *et al.*, 1988a), led to the identification of a novel type of receptor-like tyrosine phosphatase (Charbonneau *et al.*, 1988, 1989). These receptor-like molecules may themselves be central components in the transduction of as yet unidentified extra- to intracellular signals. Elucidation of the structure–function relationships of these proteins will be useful in understanding their physiological roles.

The PTPases can be divided into three classes based on their structural organization. Class I contains the low molecular mass non-receptor molecules possessing a single catalytic domain (PTP 1B, TCPTP, rat brain PTP) (Charbonneau *et al.*, 1989; Cool *et al.*, 1989; Guan *et al.*, 1990). Class II and III PTPases are receptor-like transmembrane proteins. While the sole member of Class II (HPTPβ) has a single cytoplasmic catalytic domain, Class III members (LCA, LAR, HPTPα, HPTPγ, HPTPδ, HPTPε, DPTP, DLAR) possess two repeated putative catalytic domains in the cytoplasmic region of the molecule (Charbonneau *et al.*, 1988, 1989; Streuli *et al.*, 1988, 1989; Krueger *et al.*, 1990). The catalytic domains of all PTPases share significant amino acid homology and evolutionary conservation. Certain sequence motifs are highly conserved between different PTPases as well as between repeated domains 1 and 2 within the same PTPase.

In spite of this homology, evidence that the second domain of the Class III enzymes may not have phosphatase activity comes from site-directed mutagenesis of a cysteine residue found in a highly conserved sequence (VHCSAG) in both domains 1 and 2 (Streuli *et al.*, 1989, 1990). Mutation of Cys1522 in domain 1 of LCA abolishes the activity of these PTPases towards phosphotyrosyl-MBP, -Raytide or PNPP and indicates that this amino acid is essential for activity. However, mutation of the analogous cysteine within the same conserved sequence in domain 2 has no effect on PTPase activity, suggesting that domain 2 of LCA and LAR is catalytically inactive. That catalytic activity of this second domain is not absolutely essential for receptor-like PTPase-mediated dephosphorylation is further borne out by the existence of the Class II molecule HPTPβ which has only one catalytic domain, and by the finding that two recently cloned Class III receptor-like PTPases, HPTPγ/RPTPγ and RPTPβ have aspartate in place of the otherwise invariant cysteine residue in the domain 2 VHCSAG motif (Kaplan *et al.*, 1990; Krueger *et al.*, 1990).

The function of domain 2 is therefore unclear. Before accepting that domain 2 has no intrinsic PTPase activity, other possibilities must be considered. Most simply, the second domain may have an entirely different substrate specificity from the first domain. Alternatively, the second domain may possess activity in its own right, but only in a particular context. For example, when linked to an active or even an inactive domain 1, domain 2 activity may be suppressed. Both the above explanations are attractive in view of the high amino acid homology between domains 1 and 2. While not exclusive of these possibilities, domain 2 may also function as a regulator of domain 1. In support

of this is the observation that deletion of the second domain of LAR or LCA results in altered activity and substrate specificity of domain 1 (Streuli *et al.*, 1990). Thus domain 2 may directly interact with domain 1 to modulate activity of the latter, or domain 2 could regulate substrate recognition through an ability to bind to substrate even if it is incapable of phosphoester hydrolysis.

To investigate some of these possibilities and obtain a better understanding of the role of the second putative catalytic domain, we have cloned a Class III and a Class II PTPase gene from a human placental cDNA library. DNA sequence analysis shows their respective identities with the human HPTPα and β reported by others (Krueger *et al.*, 1990; Kaplan *et al.*, 1990; Matthews *et al.*, 1990). The complete intracellular region of HPTPα as well as a number of truncated HPTPα and chimaeric HPTPα/β forms have been expressed in *Escherichia coli* and their biochemical properties examined. Here we report that the second homologous domain of HPTPα is catalytically active and has a different substrate specificity from domain 1. The functional interactions between the two domains appear to modulate and in certain cases eliminate overall PTPase activity.

Results

Catalytic activity of domain 1 and domain 2 of HPTPα

To investigate the role of the reportedly inactive second homologous domain, cDNA sequences corresponding to either the entire cytoplasmic region of HPTPα (D[1 + 2]) or to the individual domain 1 (D1) or domain 2 (D2) (Figure 1A) were cloned into the vector pGEX-KG (Guan and Dixon, 1991) and expressed in *E.coli* as glutathione-S-transferase fusion proteins. The fusion proteins were affinity purified from bacterial cell lysates using glutathione−agarose beads. The washed beads were treated with thrombin to effect the cleavage and release of the PTPase polypeptides from the glutathione-S-transferase which remained bound to the beads. The proteins routinely used in experiments were the cytoplasmic region of HPTPα containing both domains 1 and 2 (Figure 1A, line b), domain 1 alone (Figure 1A, line e) and domain 2 alone (Figure 1A, line h). These are referred to throughout the text as D[1 + 2], D1 and D2, respectively. Any variations in the forms of these proteins are specifically referred to.

Purified PTPase polypeptides containing both or either of the homologous domains (Figure 1B) were assayed for activity towards several different substrates. The D[1 + 2] polypeptide and D1 dephosphorylated PNPP (Figure 2A) phosphotyrosyl-RR-src (Figure 2B) and phosphotyrosyl-MBP (Figure 2C). In contrast, the HPTPα fragment containing only D2 had no detectable activity towards MBP, even when assayed at a 10-fold higher molar concentration than the D1 or D[1 + 2] polypeptides (Figure 2C). However, D2 dephosphorylated PNPP with significant activity comparable to that of D1 (Figure 2A) and exhibited low but detectable activity towards RR-src (Figure 2B). The dephosphorylation of RR-src by D2 was more readily observed at higher (μM) concentrations of D2 (Figure 3). A truncated form of D2 (D2 1714−2601, Figure 1A line i), expressed and purified exactly as was D2, had no detectable activity towards PNPP or RR-src (not shown). This negative control indicates that the relatively high activity

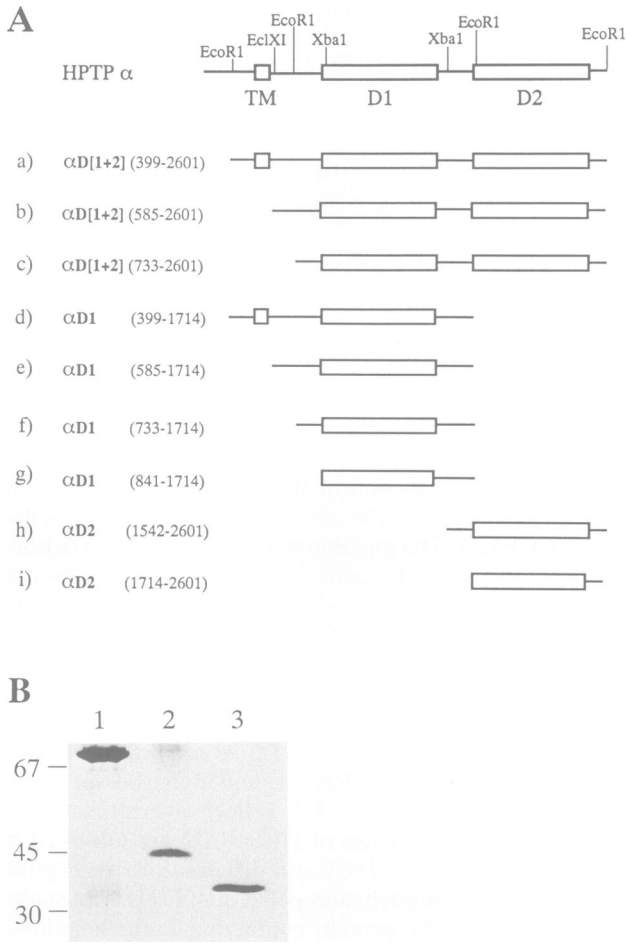

Fig. 1. Cloning, expression and purification of HPTPα fragments. **A.** Schematic diagram of portions of the HPTPα cDNA cloned into the bacterial expression vector pGEX-KG. Numbering corresponds to nucleotide sequence and follows that of Krueger *et al.* (1990). **B.** Purified PTPases obtained after expression, affinity-purification and thrombin cleavage of GST−PTPase fusion proteins. Lane 1, D[1 + 2] (585−2601); lane 2, D1 (585−1714), lane 3, D2 (1542−2601). The numbers to the left indicate the positions of molecular weight marker proteins (kDa).

of D2 towards PNPP is unlikely to be due to the presence of contaminating bacterial enzymes in the D2 preparation. Neither D[1 + 2] nor D2 could dephosphorylate phosphoseryl-casein (not shown).

The kinetic parameters of these three forms of HPTPα were determined over a range of substrate concentrations. The Lineweaver−Burk plot of PNPP dephosphorylation is shown in Figure 4. At high PNPP concentrations all the PTPases are slightly inhibited and this inhibition is very strong at high RR-src concentrations (not shown). Table I shows the kinetic constants of dephosphorylation of PNPP, MBP and RR-src. For each substrate, D1 and D[1 + 2] exhibit similar K_m values to one another, while the K_m values of D2 range from 6- (PNPP) to 50-fold (RR-src) higher. In the case of PNPP, the V_{max} of D2 is nearly 2-fold higher than that of D1. Together the maximal velocities of D1 and D2 are both equivalent to that of D[1 + 2], indicating that at high concentrations of this substrate D1 and D2 may function independently and additively. The Lineweaver−Burk plots of PNPP and RR-src dephosphorylation were linear whereas those for MBP dephosphorylation

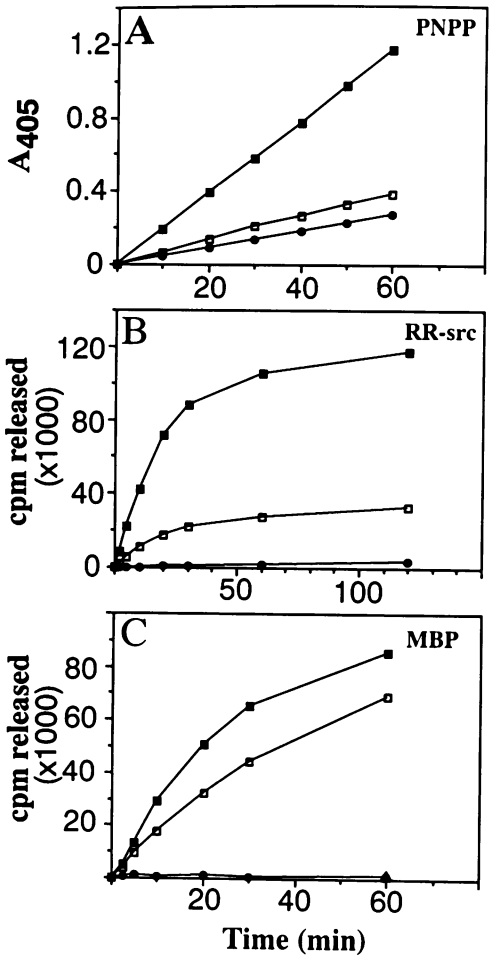

Fig. 2. Time-dependent dephosphorylation of different substrates by equimolar concentrations of D1, D2 and D[1 + 2]. The PTPases used were D1 (585–1714) (□), D2 (1542–2601) (●) and D[1 + 2] (585–2601) (■). **A.** Dephosphorylation of 2 mM PNPP by 10 nM PTPases. Reactions of 450 μl were stopped at the times indicated and absorbance at 405 nm was measured. **B.** Dephosphorylation of 5 μM RR-src by 2.5 nM PTPases. Aliquots (30 μl) of a 150 μl reaction were withdrawn at the times indicated and ^{32}P released, quantified as in Materials and methods. **C.** Dephosphorylation of 2 μM MBP by 5 nM PTPases. Aliquots (40 μl) of a 300 μl reaction were withdrawn at the times indicated and ^{32}P released, quantified as described in Materials and methods. One aliquot was taken at 60 min from a reaction containing MBP and 50 nM D2 (▲). The given concentrations of RR-src and MBP are the concentrations of phosphotyrosine present. Points shown represent the values obtained after subtracting control (minus enzyme) values.

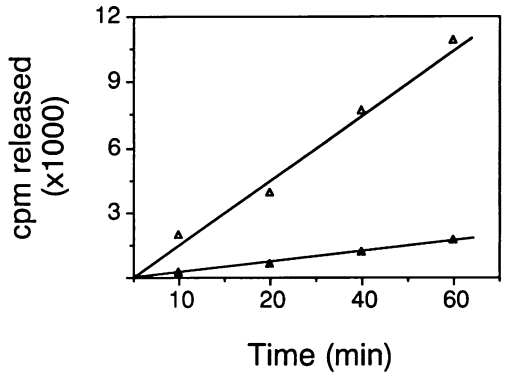

Fig. 3. Time-dependent dephosphorylation of RR-src by different concentrations of D2. At the times indicated, 30 μl aliquots were withdrawn from 150 μl reactions containing 5 μM RR-src and 250 nM (▲) or 2.5 μM D2 (1542–2601) (△). The ^{32}P-released was quantified as described in Materials and methods.

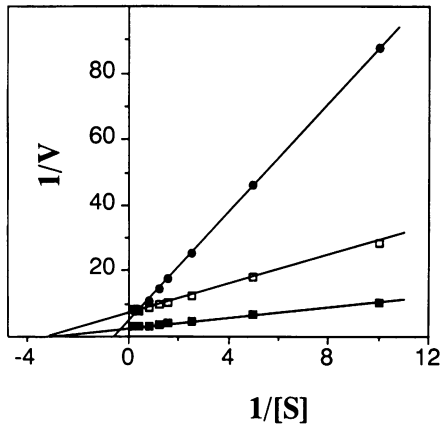

Fig. 4. Lineweaver–Burk plot of PNPP dephosphorylation by D1, D2, and D[1 + 2]. Reactions contained D1 (585–1714) (□), D2 (1542–2601) (●), or D[1 + 2] (585–2601) (■). Initial velocity (V) is in nmol/min/mg and substrate concentration [S] is in mM.

were non-linear (not shown), as has been found with this substrate and the PTPase CD45 (Tonks *et al.*, 1990). Such non-linearity often reflects the presence of multiple active sites. As it was observed with both D1 and D[1 + 2], it is unlikely that this is an effect of more than one catalytic site. The K_m and V_{max} values for MBP shown in Table I were extrapolated from the linear range of the plot at high substrate concentrations. As MBP concentration was reduced the slope of the line decreased to such an extent that it was difficult to accurately estimate the resulting low K_m and V_{max} values and so these are not given. Unlike PNPP, MBP was not a substrate for D2 (Figure 2C) and this is reflected in the similar kinetic constants obtained for D1 and D[1 + 2]. These results indicate that for certain substrates the catalytic contribution of D2 or its effect on D1 activity may be negligible. The peptide RR-src was not efficiently dephosphorylated by D2 as seen by the comparatively unfavourable kinetic constants. However, deletion of D2 may detrimentally affect the activity of the first domain since the maximal rate of activity of D1 towards RR-src is half that of D[1 + 2].

Inactivation of domain 1 abolishes activity of domain 2

The expressed forms of HPTPα (Figure 1) containing a portion of the extracellular domain, the transmembrane segment, and domains 1 and 2 D[1 + 2] (339–2601, Figure 1A, line a) or D1 alone (D1 399–1714, Figure 1A, line d) are active towards RR-src. Truncated forms of these proteins possessing most of the sequence between the transmembrane segment and domain 1 (D[1 + 2] 585–2601 and D1 585–1714, Figure 1A, lines b,e) also had comparable PTPase activity. However, further deletion of the sequence amino-terminal to the first domain (D[1 + 2] 733–2601, D1 733–1714, D1 841–1714, Figure 1A, lines c,f,g) resulted in the lack of detectable PTPase activity towards both RR-src and PNPP. Likewise, D2 possessing the immediate amino-terminal sequence (D2 1542–2601, Figure 1A, line h) was catalytically active but deletion of this region (D2 1714–2601, Figure 1A, line i) abolished

Table I. Kinetic parameters of D1, D2 and D[1 + 2] activity

Substrate	D1		D2		D[1 + 2]	
	K_m	V_{max}	K_m	V_{max}	K_m	V_{max}
PNPP	300	1420	1820	2250	290	3640
MBP	6.2	440	–	–	5.3	510
RR-src	6.2	510	290	2.5	8.3	1100

All measurements were made during the initial velocity of the reactions catalysed by D1 (nucleotides 585–1714), D2 (nucleotides 1542–2601) and D[1 + 2] (nucleotides 585–2601). Values represent data from at least duplicate experiments. Saturation was not reached with D2 and RR-src and these reactions were carried out at substrate concentrations 5-fold or more below K_m. The K_m values are expressed in μM and the V_{max} values as nmol phosphate released/min/mg phosphatase.

Table II. Effects of various compounds on the PTPase activities of D1, D2 and D[1 + 2].

Addition	Concentration	Substrates					
		PNPP			RR-src		
		D1	D2	D[1 + 2]	D1	D2	D[1 + 2]
$MnCl_2$	1 mM	87	87	63	63	96	64
$MgCl_2$	1 mM	102	96	100	94	106	86
$CaCl_2$	1 mM	98	100	102	85	99	86
$ZnCl_2$	100 μM	59	63	65	75	94	76
	1 mM	27	1	2	3	10	1
EDTA	2 mM	82	47	69	ND	ND	ND
	5 mM	55	21	46	112	76	100
Na_3VO_4	100 μM	4	12	6	8	78	19
Spermine	2 mM	72	75	94	55	66	58
Heparin	0.5 μg/ml	87	95	99	93	115	95
	5 μg/ml	72	15	103	97	98	92
	50 μg/ml	32	2	44	136	5	364
Poly(Glu4:Tyr1)	5 μM	69	9	105	86	73	89
	50 μM	47	0	133	88	5	156

Reactions catalysed by D1 (nucleotides 585–1714), D2 (nucleotides 1542–2601) and D[1 + 2] (nucleotides 585–2601) were stopped while the rate of dephosphorylation was linear with time. The activity of each enzyme in the absence of additions was taken as 100%. The reactions contained 5 mM PNPP or 5 μM RR-src with the exception of those with D2 and RR-src which contained 25 μM RR-src. The values represent averaged data from at least two experiments.

the PTPase activity of domain 2. Thus the regions amino-terminal to each homologous repeat are critical for PTPase function.

As mentioned above, the removal of sequence 585–732 from D[1 + 2] (Figure 1A, compare lines b and c) results in the loss of PTPase activity, even though this form of HPTPα contains D2 and D2 amino-terminal sequence which alone are sufficient for PNPP hydrolysis. Since loss of sequence 585–732 from D1 alone (Figure 1A, compare lines e and f) also results in an inactive form of D1 (D1 733–1714, Figure 1A, line f), it is likely that D1 in the similarly truncated D[1 + 2] form (733–2601, Figure 1A, line c) is inactive. Therefore it appears that inactivation of domain 1 results in the suppression of the intrinsic PTPase activity of the second homologous domain, whereas the loss of D1 does not.

Effects of various compounds on domain 1 and domain 2 PTPase activities

The PTPase activities of D1, D2 and D[1 + 2] towards PNPP and RR-src were characterized in terms of the

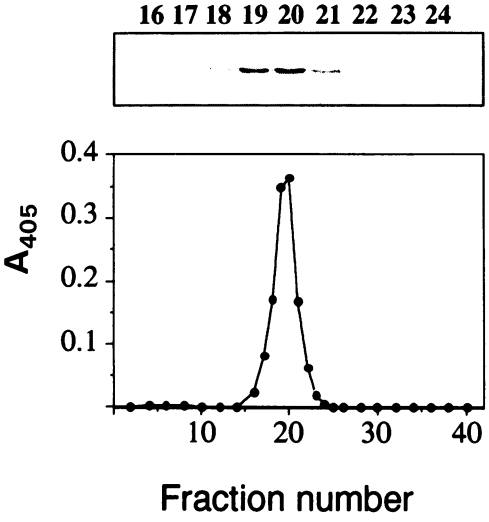

Fig. 5. Zn^{2+}-affinity adsorption of D2 PTPase. The D2 (1542–2601) PTPase (130 μg) was applied to a Zn^{2+}-affinity column as described in Materials and methods. The PNPP phosphatase activity profile of fractions eluted from the column with EDTA is shown. The boxed area above the activity profile shows the corresponding presence of D2 in the eluted fractions (numbered above the box) analysed by SDS–PAGE.

responses to several possible effectors (Table II). The divalent cations Mg^{2+} and Ca^{2+} had little effect on PTPase activities and Mn^{2+} was in certain cases slightly inhibitory. The chelator EDTA, which has been noted to stimulate or to have little effect on the CD45 or PTP 1B enzymes (Tonks *et al.*, 1988b, 1990; Pallen *et al.*, 1991) was an effective inhibitor of the three forms of HPTPα towards PNPP but less so towards RR-src. At 100 μM, the typical PTPase inhibitor vanadate strongly inhibited D1, D2 and D[1 + 2] activities although D2 activity towards RR-src appeared less susceptible to vanadate inhibition.

At 1 mM, zinc was an effective inhibitor of all the PTPases. The use of Zn^{2+}-affinity chromatography to purify PTPases (Horlein *et al.*, 1982; Shriner and Brautigan, 1984) was developed based on the ability of micromolar concentrations of this cation to potently inhibit the activity of this class of enzymes. To determine whether the second domain of HPTPα possesses the characteristically high affinity for Zn^{2+} of other such enzymes, we applied D2 to a Zn^{2+}-iminodiacetic acid–agarose column. The column was extensively washed and then eluted with EDTA. No protein or PTPase activity was detected in the breakthrough or wash fractions. A peak of activity corresponding to the presence of D2 was found in the EDTA-eluted fractions (Figure 5), demonstrating that the catalytically active second domain has the necessary sequence(s) for specific interaction with Zn^{2+}.

The polyanionic compounds heparin and poly (Glu4, Tyr1), which inhibit the receptor-like PTPase CD45 and strongly inhibit the non-receptor PTP 1B (Tonks *et al.*, 1990), differentially affected the three forms of HPTPα. At the highest concentrations of these compounds, the activity of D2 towards PNPP and RR-src was nearly abolished, D1 was less strongly inhibited, and D[1 + 2] activity was unexpectedly stimulated about 3–4 fold (Table II). Thus the effects of these compounds on the individual domains clearly do not pertain to those observed on the single polypeptide possessing both domains.

```
D1   ENKEKNRYVNILPYDHSRVHLTPVEGVPDSDYINASFINGYQEKNKF
D2   ANMKKNRVLQIIPYEFNRVIIPVKRGEENTDYVNASFIDGYRQKDSY
          Y       D      L

D1   IAAQGPKEETVNDFWRMIWEQNTATIVMVTNLKERKECKCAQYWPD
D2   IASQGPLLHTIEDFWRMIWEWKSCSIVMLTELEERGQEKCAQYWPS

D1   QGCWTYGNIRVSVEDVTVLVDYTVRKFCIQQVGDMTNRKPQRLITQFH
D2   DGLVSYGDITVELKKEEECESYTVR----DLLVTNTRENKSRQIRQFH

D1   FTSWPDFGVPFTPIGMLKFLKKVKACNPQYA-GAIVVHCSAGVGRTG
D2   FHGWPEVGIPSDGKGMISIIAAVQKQQQQSGNHPITVHCSAGAGRTG
                                      G

D1   TFVVIDAMLDMMHTERKVDVYGFVSRIRAQRCQNVQTDMQYVFIYQ
D2   TFCALSTVLERVKAEGILDVFQTVKSLRLQRPHMVQTLEQYEFCYK
           D
```

Fig. 6. Alignment of the core amino acid sequences of HPTPα domain 1 and domain 2. Amino acid residues which are identical between the two domains (domain 1, D1; domain 2, D2) are in bold type. Residues in bold type shown below the D2 sequence are those which are identical in the domain 1 sequences of 12 other PTPases (PTP 1B, TCPTP, LAR, LCA, DLAR, DPTP, and HPTP members α, β, γ, δ, ε, ζ) (Krueger *et al.*, 1990) but which are not identical in the domain 2 sequence of HPTPα.

Chimaeric HPTPα domain 1/2 and HPTPα/β PTPases

Domain 1 and domain 2 of HPTPα have 42% amino acid identity to one another (Figure 6), yet in spite of this high degree of conservation have distinct substrate specificities. Alignment of the D2 sequence of HPTPα with the D1 sequences of all other known PTPases revealed five amino acids in D2 which do not correspond to the absolutely conserved residues at these positions in the D1 sequences. We investigated if any particular region of D2 might contribute to its low activity towards RR-src by shuffling various pieces of D1 and D2 and their appropriate amino-terminal regions to create single domain D1/2 chimaerae which were then tested for ability to dephosphorylate RR-src. Since D1 and D2 share regions of identical cDNA sequence within which are located conserved restriction sites, conveniently spaced sites were used as points at which to cut and religate the D1 and D2 fragments (Figure 7, lines a,b). To examine the feasibility of this approach we first tested whether we could reconstitute active chimaeric domains from pieces of two different domains with intrinsic PTPase activity, namely D1 and the catalytic domain of HPTPβ (Figure 7, line c). HPTPβ is a highly active PTPase with a 2-fold lower K_m and a 20-fold higher V_{max} for RR-src than D[1 + 2] (unpublished results).

Four chimaeric forms of D1 and HPTPβ were constructed and expressed in *E.coli*. Exchanging the carboxyl-terminal regions of domain 1 of HPTPα and of the catalytic domain of HPTPβ along with the accompanying 'tail' sequences of each domain resulted in active PTPases but with generally lower activity (Figure 7, lines d,g). Replacing the region amino-terminal to the HPTPβ catalytic domain with that amino-terminal to D1 or vice versa (Figure 7, lines e,f) resulted in an enzyme with lower activity than HPTPβ. Thus not only the presence or absence of this upstream region (see above) but also its nature can affect the activity of the catalytic domain to which it is adjacent. Nevertheless, all the D1/β chimaerae were active towards RR-src.

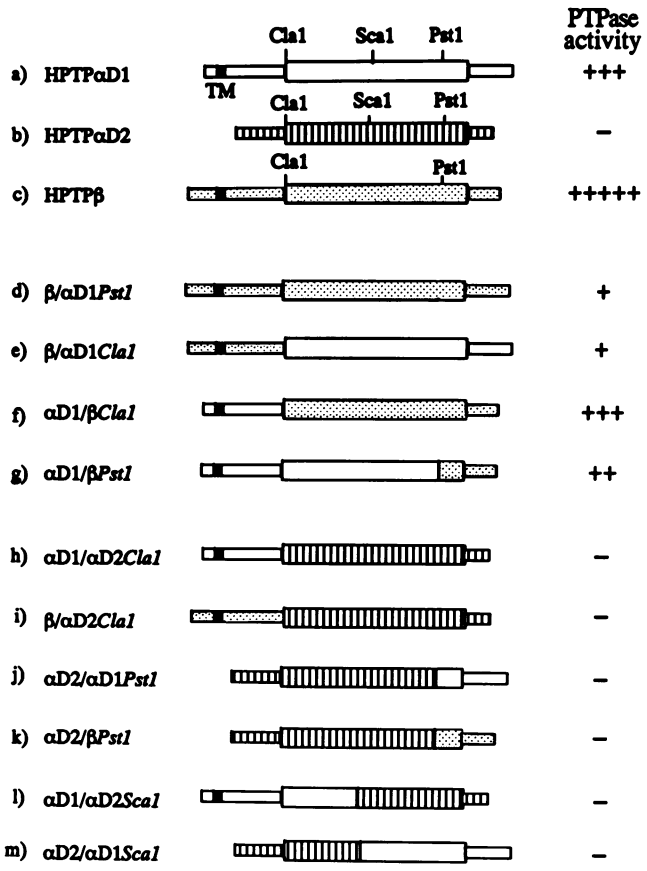

Fig. 7. Schematic diagram of the regions of HPTPα and HPTPβ utilized to produce chimaeric PTPases. Positions of the restriction sites used to create chimaeric molecules are shown in (**a**) D1 and adjoining amino- and carboxy-terminal regions, (**b**) D2 and adjoining amino- and carboxy-terminal regions and (**c**) the catalytic domain of HPTPβ and adjoining amino- and carboxy-terminal sequences. Chimaeric D1/β molecules are shown in (**d**)–(**g**) and chimaeric D1/D2 and D2/β molecules are shown in (**h**)–(**m**). Relative PTPase activities towards RR-src of bacterial lysates containing approximately equal amounts of expressed fusion protein are indicated by plus and minus (undetectable activity) signs.

In contrast, none of the D1/2 or the D2/β chimaerae possessed detectable RR-src PTPase activity. The regions amino-terminal to D1 or to the β catalytic domain did not activate D2 (Figure 7, lines h,i). The carboxy-terminal regions of D1 or the β domain and their attached 'tail' sequences could not activate the ~80% of D2 to which they were attached (Figure 7, lines j,k). Substitution of the amino-terminal region and first half of D2 with that of D1 and vice versa (Figure 7, lines l,m) did not result in activity towards RR-src.

Discussion

The second homologous domain of the CD45 and LAR PTPases has been reported to be catalytically inactive (Streuli *et al.*, 1990), but perhaps to act as a regulator of the activity of domain 1. While investigating the function of the two domains of HPTPα, we found that the second domain of this PTPase is catalytically active. Dissection of HPTPα and expression of the isolated domain 1 (D1) or domain 2 (D2) shows that each domain contains a functional active site with intrinsic PTPase activity. Domain 1 and domain 2 exhibit distinct substrate specificities as is evident from the kinetic

characterization of their activities towards the three substrates tested, with D1 acting on MBP ~ RR-src > PNPP whereas D2 favours PNPP ⋙ RR-src > MBP. The high degree of sequence conservation between D1 and D2 suggests that a particular region of (or adjacent to) D2 which is distinct from that in D1, or even a single residue change in D2, could account for the low or lack of activity of D2 towards RR-src and MBP. However, the substitution of portions of D1 for their D2 counterparts to form chimaeric D1/2 domains did not reproduce the D1-like RR-src phosphatase activity. The sequence immediately amino-terminal to either D1 or D2 is important for activity since its deletion results in inactive domains. This sequence could also possibly contribute to substrate specificity. Chimaeric proteins containing the regions amino-terminal to D1 or to the active catalytic domain of HPTPβ in conjunction with D2 did not alter the low specificity of D2 for RR-src. These results indicate that the different substrate specificities of D1 and D2 are not determined by a particular amino acid substitution, distinct subdomain, or amino-terminal region but rather may reflect the total nature of D1 and D2 dissimilarities.

The interaction between D1 and D2 appears to be complex. Deletion of D2 seemingly has a negative effect on the RR-src phosphatase activity of D1 as indicated by the lower V_{max} of D1 compared to that of D[1 + 2] (Table I), by the observation that D1 activity has almost reached a plateau at ~10–15% substrate dephosphorylation in contrast to a maximum of at least 40% dephosphorylation attained by D[1 + 2] (Figure 2B), and by the finding that D2 catalyses RR-src dephosphorylation very inefficiently. However within the context of the holoenzyme, the contribution of D2 to HPTPα-catalysed RR-src hydrolysis is unknown and so it is difficult to conclude whether D2 regulates D1, or whether D1 regulates D2. The latter case could be envisaged if D1 acts as a positive modulator of D2 and the loss of D1 thus removes the potential for higher D2 activity.

Evidence supporting the possibility that D1 can regulate D2 activity comes from our studies of the effects of truncation of the sequence amino-terminal to D1. Successive shortening of this region (Figure 1A, lines a–g) results in the loss of activity of the D1 enzyme and of the D[1 + 2] enzyme. The D[1 + 2] PTPase contains all the sequence necessary for intrinsic D2 PTPase activity, suggesting that inactivation of D1 either suppresses the activity of D2 or results in the loss of ability of D1 to activate D2. Streuli *et al.* (1990) have reported that mutation of a conserved cysteine residue in domain 1 of the double domain PTPases CD45 and LAR abolishes enzyme activity, while mutation of the analogous cysteine residue in domain 2 has little effect on activity. They concluded that D2 does not possess intrinsic PTPase activity since it is unlikely that the mutation in D1 could result in a conformational change in D2 leading to D2 inactivation. An alternative explanation in light of the knowledge that HPTPα D2 has intrinsic PTPase activity is that an active D1 can induce a conformational change in D2 leading to D2 activation. This model predicts that in the holoenzyme, if D1 is not active towards a substrate then neither will be D2. Deletion of D1 could relieve the negative conformational constraints upon D2 activity.

Our studies have established that the two homologous domains of HPTPα each contain a functional active site. It is likely that the second domain of other such PTPases is also catalytically active, with the possible exceptions of HPTPγ/RPTPγ and RPTPβ which lack a highly conserved cysteine residue in domain 2 (Kaplan *et al.*, 1990; Krueger *et al.*, 1990). The receptor-like nature of HPTPα, in conjunction with the presence of two catalytic domains, indicates that HPTPα activity could be subject to regulation by multiple mechanisms. Events that may modulate the activity or specificity of this PTPase include the binding of putative ligands to the extracellular region, the interaction of protein regulators or other effector molecules with the cytoplasmic portion of the molecule, substrate binding, and the interaction of the two catalytic domains. The differences in *in vitro* substrate specificities and responses to effectors of domains 1 and 2 suggest that these active sites could be subject to differential regulation and fulfill distinct functions in the cell.

Materials and methods

Molecular cloning of HPTPα and HPTPβ

A pair of degenerate oligonucleotides corresponding to the conserved sequences KCAQYWP and VHCSAGVGR (Krueger *et al.*, 1990) of PTPases were synthesized and used in a PCR reaction to amplify DNA sequences from a human placenta cDNA/λgt11 library (obtained from Clontech, USA). The amplified DNA fragments were cloned into M13mp18 (Yanisch-Perron *et al.*, 1985) and a large number of clones were subjected to DNA sequencing analysis. DNA from the clones containing sequences homologous to but distinct from the known (at that time) PTPases were used to screen the placenta cDNA library using the procedure provided by the manufacturer. Positive clones were grown and DNA was prepared as described (Sambrook *et al.*, 1989). The cDNA from each clone was cut out with *Eco*RI and subcloned into M13mp18 and Bluescript for DNA sequencing. Sequencing analysis showed that two groups of cDNA clones had respective identities with HPTPα and HPTPβ (Kaplan *et al.*, 1990; Krueger *et al.*, 1990).

Plasmid constructions

HPTPα and β cDNA was cut with several restriction enzymes and DNA fragments coding for different domains of the phosphatase were inserted into the bacterial expression vector pGEX-KG (Guan and Dixon, 1991) at appropriate sites to ensure a correct reading frame. All the plasmids were transformed into transformation competent DH5αF′ cells (BRL).

Bacterial extracts and purification of GST–PTPase fusion proteins

A single DH5αF′ colony carrying the desired pGEX-KG-PTPase was grown overnight in LB medium supplemented with 100 μg/ml ampicillin. The overnight culture was inoculated at a 1:20 dilution into LB medium containing 100 μg/ml ampicillin and grown at 37°C with vigorous shaking for 1 h before IPTG was added to 0.5 mM. The culture was shaken for three more hours at either 25°C for the fusion proteins with poor solubility or at 37°C for soluble fusion proteins. Cells were spun down and washed once with MTPBS (150 mM NaCl, 16 mM Na_2HPO_4, 4 mM NaH_2PO_4, pH 7.3) and resuspended in 1/50 to 1/100 culture volume of MTPBS containing 1 mg/ml lysozyme, 1% Triton X-100 and 20 μg/ml of the protease inhibitor aprotinin (Sigma). Cells were incubated at 37°C for 10 min and then chilled on ice followed by 1 min sonication (three times) at full power on a Microson ultrasonic cell disruptor (Model XL) at 4°C. The cell lysate was kept on ice for 30 min. The lysate was either directly assayed for PTPase activity or spun at 10 000 *g* for 10 min at 4°C and the supernatant was collected for further purification. The affinity purification of GST-PTPase fusion proteins was carried out essentially as described (Guan and Dixon, 1991). The supernatant (10 ml) from a 1 l culture was mixed with 1 ml of a 50% suspension of glutathione–agarose beads (Sigma) in MTPBS and incubated at 4°C for 1 h on a rotary mixer. The beads were collected by brief centrifugation and washed five times with 15 ml cold MTPBS containing 1 mM DTT. Finally the beads were resuspended in 500 μl thrombin cleavage buffer containing 50 mM Tris (pH 8.0), 150 mM NaCl, 2.5 mM $CaCl_2$, 0.1% 2-mercaptoethanol and 5 μg thrombin (Sigma) and incubated at 25°C for 30 min. The beads were spun down and the supernatant containing the phosphatase was collected, glycerol added to 20%, and stored at −20°C. Detectable fusion proteins were expressed from all constructs.

If bacterial lysates were to be assayed for PTPase activity, the relative amounts of expressed fusion proteins in different lysates were calibrated as follows. Aliquots of the bacterial cell suspension were taken prior to the addition of lysozyme, mixed with Laemmli sample buffer, and electrophoresed on SDS–polyacrylamide gels. The gels were stained with Coomassie blue and the amount of fusion protein quantified in arbitrary units of integrated intensity by densitometric scanning and computerized whole band analysis using a Visage 200 Image analysis system (BioImage Products, Michigan). Volumes of different lysates were then selected so as to contain approximately equal amounts of expressed fusion protein and assayed for PTPase activity.

The amounts of purified phosphatases recovered after thrombin cleavage were quantified as follows. Known volumes of the phosphatase preparations were electrophoresed on duplicate SDS–polyacrylamide gels alongside several different known amounts of a protein standard (thrombin). After staining with Coomassie blue, the gels were scanned and the integrated intensity of the stained bands representing phosphatase or thrombin quantified by computerized densitometry and whole band analysis using the Visage 2000 Image analysis system. A standard curve of integrated intensity versus μg thrombin was used to estimate the μg phosphatase loaded on the gels. This method of protein estimation was found to be highly reproducible when duplicate or triplicate samples were analysed, and eliminated overestimation of phosphatase concentration since small amounts of glutathione-S-transferase, thrombin, or minor contaminants sometimes present in the preparations were not included in the quantification. The purity of the phosphatase preparations ranged from 80–85% (D[1 + 2]), 75–80% (D1) and 90–95% (D2) as determined by densitometry. Average ($n \geq 5$) yields of purified D[1 + 2], D1 and D2 from a 1 l culture were 30, 30 and 750 μg phosphatase, respectively.

Phosphatase substrates

The RR-src peptide (custom synthesized by Multiple Peptide Systems, Inc.) representing an autophosphorylation site of the src kinase (RRLIEDAEY-AARG), was phosphorylated by EGF receptor kinase as previously described (Goris *et al.*, 1988). Myelin basic protein from bovine brain (Sigma) was phosphorylated by v-abl tyrosine kinase (Oncogene Science) essentially as described by the suppliers. Reactions contained 100 μM ATP (2000–3000 c.p.m./pmol) and were carried out at 30°C for 16 h. Reactions were terminated and the precipitated protein washed as described by Streuli *et al.* (1990) and then resuspended in 0.1 M HEPES (pH 7.5). PNPP was purchased from Sigma. Casein (Sigma) was phosphorylated in a reaction mixture containing 1 mg/ml casein, 70 U/ml catalytic subunit of bovine heart cAMP-dependent protein kinase (Sigma), 40 mM Tris (pH 7.5), 20 mM $MgCl_2$, and 0.5 mM ATP (1000 c.p.m./pmol). After incubation at 30°C for 40 min the reaction was stopped by the addition of TCA to 5%. Precipitated protein was washed three times with 20% (w/v) TCA and resuspended in 25 mM Mes (pH 6.5).

Assays of phosphatase activity

All PTPase assays were carried out at 30°C. Due to the different extents of substoichiometric phosphorylation of RR-src and MBP, the concentrations of these substrates referred to in the text indicate the concentration of phosphotyrosine present in the reaction. PTPase activity towards RR-src or MBP was assayed in reaction mixtures containing 50 mM Mes (pH 6.0), 0.5 mg/ml BSA, 0.5 mM DTT and the indicated amounts of substrate. Reactions were terminated by the addition of a 10-fold excess volume of 5% (w/v) TCA/100 mM potassium phosphate and the ^{32}P released, quantified as described previously (Chan *et al.*, 1986). PTPase activity towards PNPP was assayed in 450 μl reactions containing 50 mM sodium acetate (pH 5.5), 0.5 mg/ml BSA, 0.5 mM DTT, and PNPP as indicated. Reactions were terminated by the addition of 50 μl 13% K_2HPO_4. The amount of the product *p*-nitrophenol was calculated from the absorbance of the stopped reaction at 405 nm using the extinction coefficient of 1.8×10^{-4} cm^{-1}. Non-enzymatic hydrolysis of PNPP was accounted for by performing appropriate control reactions without added enzyme, and this absorbance was subtracted from that attained in the presence of enzyme. PTPase activity towards casein was assayed in reactions containing 1 μM phosphoseryl-casein, 50 mM Mes (pH 6.0), 0.5 mg/ml BSA, 0.5 mM DTT and 20 nM D[1 + 2] or 1 μM D2. Reactions were stopped after 10, 30 and 60 min by the addition of TCA to 10%. After incubation on ice for 10 min the mixtures were microfuged and a portion of the supernatant counted for ^{32}P.

Zn^{2+}-affinity chromatography

The chelating Sepharose 6B (Pharmacia) was prepared and charged with Zn^{2+} according to the manufacturer's instructions. Chromatography was carried out at 4°C. A column (1.0 × 7.0 cm) was equilibrated with 20 mM HEPES, 50 mM NaCl, pH 7.5. The purified D2 protein from HPTPα (130 μg) was dialysed overnight against equilibration buffer, applied to the column and the column washed with 8 vol of equilibration buffer before elution with equilibration buffer containing 40 mM EDTA. The column was run at a flow rate of 0.4 ml/min and 0.4 ml fractions were collected. One-tenth volume of the fractions was used to assay PTPase activity or analysed by SDS–PAGE.

Acknowledgements

We thank K.Guan and J.E.Dixon for their generous gift of the vector pGEX-KG, Y.H.Tan and W.Chia for critical reading of the manuscript, S.Brenner for interesting discussions, C.Kong for assistance with the densitometric analyses, M.L.Chua for excellent secretarial assistance and F.Leong for photography.

References

Chan,C.P., Gallis,B., Blumenthal,D.K., Pallen,C.J., Wang,J.H. and Krebs,E.G. (1986) *J. Biol. Chem.*, **261**, 9890–9895.
Charbonneau,H., Tonks,N.K., Walsh,K.A. and Fischer,E.H. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 7182–7186.
Charbonneau,H., Tonks,N.K., Kumar,S., Diltz,C.D., Harrylock,M., Cool,D.E., Krebs,E.G., Fisher,E.H. and Walsh,K.A. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 5252–5256.
Cool,D.E., Tonks,N.K., Charbonneau,H., Walsh,K.A., Fisher,E.H. and Krebs,E.G. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 5257–5261.
Dunphy,W.G. and Newport,J.W. (1989) *Cell*, **58**, 181–191.
Goris,J., Pallen,C.J., Parker,P.J., Herman,J., Waterfield,M.D. and Merlevede,W. (1988) *Biochem. J.*, **256**, 1029–1034.
Gould,K.L. and Nurse,P. (1989) *Nature*, **342**, 39–45.
Guan,K., Haun,R.S., Watson,S.J., Geahlen,R.L. and Dixon,J.E. (1990) *Proc. Natl. Acad. Sci. USA*, **87**, 1501–1505.
Guan,K. and Dixon,J.E. (1991) *Analyt. Biochem.*, **192**, 262–267.
Horlein,D., Gallis,B., Brautigan,D.L. and Bornstein,P. (1982) *Biochemistry*, **21**, 5577–5584.
Kaplan,R., Morse,B., Huebner,K., Croce,C., Howk,P., Ravera,M., Ricca,G., Jaye,M. and Schlessinger,J. (1990) *Proc. Natl. Acad. Sci. USA*, **87**, 7000–7004.
Krueger,N.X., Streuli,M. and Saito,H. (1990) *EMBO J.*, **9**, 3241–3252.
Matthews,R.J., Cahir,E.D. and Thomas,M.L. (1990) *Proc. Natl. Acad. Sci. USA*, **87**, 4444–4448.
Morla,A.O., Draetta,G., Beach,D. and Wang,J.Y.J. (1989) *Cell*, **58**, 193–204.
Pallen,C.J., Lai,D.S.Y., Chia,H.P., Boulet,I. and Tong,P.H. (1991) *Biochem. J.*, **276**, 315–323.
Sambrook,J., Fritsch,E.F. and Maniatis,T. (1989) *Molecular Cloning: A Laboratory Manual*. 2nd edition. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, p. 260.
Shriner,C.L. and Brautigan,D.L. (1984) *J. Biol. Chem.*, **259**, 11383–11390.
Streuli,M., Krueger,N.X., Hall,L.R., Schlossman,S.F. and Saito,H. (1988) *J. Exp. Med.*, **168**, 1523–1530.
Streuli,M., Krueger,N.X., Tsai,A.Y.M. and Saito,H. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 8698–8702.
Streuli,M., Krueger,N.X., Thai,T., Tang,M. and Saito,H. (1990) *EMBO J.*, **9**, 2399–2407.
Tonks,N.K., Diltz,C.D. and Fischer,E.H. (1988a) *J. Biol. Chem.*, **263**, 6722–6730.
Tonks,N.K., Diltz,C.D. and Fischer,E.H. (1988b) *J. Biol. Chem.*, **263**, 6731–6737.
Tonks,N.K., Diltz,C.D. and Fischer,E.H. (1990) *J. Biol. Chem.*, **265**, 10674–10680.
Yanisch-Perron,C., Vieira,J. and Messing,J. (1985) *Gene*, **33**, 103–119.

Received on July 3, 1991; revised on July 29, 1991