Protein tyrosine phosphatase PTP-S binds to the juxtamembrane region of the hepatocyte growth factor receptor Met

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We reported previously that a protein tyrosine phosphatase (PTP) activity is associated with the immunoprecipitated hepatocyte growth factor (HGF) receptor, also known as Met. The activity increased reversibly when Met was stimulated by HGF and decreased when Met was inactivated by PMA. To identify the PTP-binding region, we used deletion mutants of the receptor β -subunit. The PTP activity did not associate with Tpr-Met, a construct containing residues 1010–1390 of Met fused to Tpr. In contrast, PTP activity was present when the expressed protein contained the full juxtamembrane region (residues 956–1390 of Met) or part of this region (residues 957–1390 or 995–1390), indicating that the PTP-binding region is between residues 995 and 1009. This region includes Tyr¹⁰⁰³, a site involved in Met downstream signalling. Incubation of Met immunoprecipitated from GTL-16 cells with an 8-mer phosphopeptide derived from residues 1003–1010 induced a marked decrease in the associated

PTP activity, suggesting that the peptide reproduced the PTP-binding region. Mutation of Glu, Asp or Arg at positions –4, –1 or +1 respectively relative to Tyr¹⁰⁰³ in a 9-mer peptide (residues 999–1007) abolished the ability of the peptide to decrease the PTP activity associated with Met. Phosphorylation of Tyr¹⁰⁰³ was not required for PTP binding, since: (1) both phospho- and dephospho-peptides on a solid bead bound PTP activity from a GTL-16 cell extract, and (2) PTP activity was associated with a Met deletion mutant lacking residues 1–955 in which Tyr¹⁰⁰³ had been changed into Phe. In order to partially purify the PTP from the GTL-16 cell extract, an affinity column was prepared using the Met-derived peptide comprising residues 998–1007. Less than 0.1 % of the total cellular PTP was retained by the column, and was eluted with low salt concentrations. Using antibodies, this PTP was identified as PTP-S, a soluble PTP present in epithelial cells and fibroblasts.

INTRODUCTION

The receptors of growth factors are generally protein tyrosine kinases (PTKs) which undergo autophosphorylation on receptor binding and subsequently phosphorylate various substrates involved in receptor signalling. Protein tyrosine phosphatases (PTPs) are required so that such phosphorylations are reversible, and consequently co-ordination between PTKs and PTPs is critical (e.g. see [1]).

It is now clear that two kinds of function are carried out by the PTPs that act in concert with receptor PTKs. The first of these is the positive transduction of a signal. This is accomplished by PTPs that bind to the phosphorylated receptor, or to its adaptors, and thus become functional towards their substrates. Such a mechanism was first described in the case of corkscrew, a positive component of the Torso differentiation pathway in *Drosophila* [2]. It is now clear that SHP2 [a Src homology 2 (SH2) domain-containing PTP] generally has a positive effect on cellular signalling [3]. Another example is the overexpression of PTP α , which results in dephosphorylation and activation of the Src kinase [4]. The second function of PTPs is the negative regulation of signalling pathways. PTPs are required to dephosphorylate both the receptor PTK and its substrates, thus contributing to bringing an activated system back to its basal state. Such PTPs may be either receptor-like or soluble PTPs, and include SHP1 [3]. One example of such a mechanism is the decrease in the phosphorylation of the insulin receptor, insulin receptor substrate-1 and SHC (protein with homology to Src and collagen) in hepatoma cells overexpressing the PTP LAR ('leucocyte common antigen-related') [5]. Opposite effects were obtained when the expression of LAR was suppressed with antisense mRNA [6].

With epidermal growth factor and hepatocyte growth factor (HGF), a decrease in the level of LAR increased the phosphorylation of the receptor and of several of its transducers [6]. It is clear from these results that the same PTP may work in more than one receptor signalling pathway. In addition, given the number of phosphorylated sites involved, more than one PTP can be expected to participate in the signalling from a single receptor type.

PTPs are found in soluble, membrane and cytoskeletal fractions. Their regulatory mechanisms include interaction with regulatory proteins, phosphorylation and ligand binding or cell-cell interactions (in the case of receptor PTPs). All such events may result in activity modulation or in PTP relocation (e.g. see [7–9] for reviews).

The HGF receptor also called Met (see [10] for a review), consists of a 50 kDa α -subunit exposed on the cell surface [11] and a 145 kDa transmembrane β -subunit [12,13] endowed with tyrosine kinase activity. The intracellular domain of the β -subunit (residues 956–1390) is further divided into juxtamembrane (residues 956–1093), catalytic and C-terminal domains [12–15]. In addition to the tyrosine autophosphorylation sites, the intracellular domain also includes serine and threonine residues that may undergo phosphorylation [16]. The Tyr residues that serve as docking sites for SH2-containing transducers are located in the C-terminal tail [17].

Previously we reported that a PTP activity associated with Met could be immunoprecipitated from various cell lines. Such activity increased reversibly when Met was stimulated by HGF and decreased when Met was inactivated by PMA treatment *in vivo* [18]. This was the first example of a receptor-associated PTP that was stimulated by the receptor ligand. However, in spite of

Abbreviations used: PTP, protein tyrosine phosphatase; PTK, protein tyrosine kinase; HGF, hepatocyte growth factor; SH2, Src homology 2.

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extensive research, using antibodies against several receptor-like and soluble PTPs (including PTP α , LAR, RPTP β , PTP1B, TC-PTP, HEPTP, SAP-1, PTP36, α L1A, PAC1 and hVH-3), (see [3,7] for PTP nomenclature) the PTP involved was not identified. Also, in several experiments using immunoprecipitated or recombinant proteins, we did not find any evidence for a direct interaction between SHP2, or SHP1, and Met. Recently, SHP2 was demonstrated instead to bind to GAB1, an insulin-receptor substrate-like adaptor protein, once this has been phosphorylated by activated Met [19].

In the present work we used structural and biochemical approaches that led first to identification of the region of Met that binds the PTP, and then to the isolation and identification of this PTP as PTP-S.

MATERIALS AND METHODS

Materials

ATP, PMSF, benzamidine, Protein A-Sepharose, Protein A-peroxidase, molecular mass markers, media and reagents for cell culture were purchased from Sigma. T7 polymerase and the CellPfect transfection kit were from Pharmacia Biotech. [32 P]ATP, Hyperfilms-ECL and ECL reagents were from Amersham. Chemicals for electrophoresis were from Bio-Rad. Triton X-100 was from Boehringer. Immobilon-P was from Millipore. The UltraLink Immobilized NeutrAvidin kit was from Pierce.

The 8-mer phosphopeptides derived from the juxtamembrane region of Met reproduced residues 971–978, 1003–1010 and 1026–1033 [20]. The 9-mer peptide derived from the juxtamembrane region of Met (residues 999–1007) and the three mutated 9-mer peptides were from Genosys. The biotinylated peptide 6-biotinamidoesanoil-(Gly) $_5$ -Asn-Glu-Ser-Val-Asp-Tyr-Arg-Ala-Thr-Phe, reproducing the region encompassing residues 998–1007 of Met, was synthesized in its phosphorylated and non-phosphorylated forms by Fmoc (fluoren-9-ylmethoxycarbonyl) chemistry and purified by HPLC (> 99%) (E. Vigna, A. Bardelli and P. M. Comoglio, unpublished work; details available on application to the author). The unrelated phosphopeptide Pro-Glu-Gly-Asp-Tyr(PO $_3$ H $_2$)-Glu-Glu-Val-Leu-Glu, used as a control, was a gift from Dr. O. Marin and Dr. F. Meggio (University of Padova, Italy). The anti-PTP-S monoclonal antibodies (hybridoma supernatant G11) [21] were a gift from Dr. G. Swarup (University of Hyderabad, India).

Cell lines and constructs

NIH-3T3 cells permanently expressing various deletion mutants of the Met β -subunit (Tpr-Met, cyto-1 and cyto-2; see Table 1 for details of constructs) or the full-length construct [15], GTL-16 cells and COS-1 cells were grown in Dulbecco's modified Eagle's medium containing 10% (v/v) fetal calf serum on 150-mm plates and lysed as previously described [18]. Tpr-juxta-Met constructs, either wild-type or carrying the Tyr 1003 $\rightarrow$ Phe mutation, were obtained by PCR amplification and cloned into the pMT2 expression vector, as will be described elsewhere (E. Vigna, A. Bardelli and P. M. Comoglio, unpublished work; details available on application to the author). The fidelity of the construct was verified by double-strand DNA sequencing with T7 polymerase. Transfection into COS-1 cells was performed using the calcium phosphate CellPfect kit.

Immunoprecipitation of Met and assay of the associated PTP activity

Met was immunoprecipitated from 2.5–3.5 mg of cell extract with monoclonal antibodies and Protein A-Sepharose pre-coated

with rat anti-(mouse IgG). Antibodies directed to the N-terminus (DO-24) were used for the wild-type receptor, and antibodies directed to the C-terminus (DQ-13) were used for the β -subunit constructs and for the experiments depicted in Figure 1 and Table 2. The immunocomplexes were washed in 50 mM Hepes, pH 7.4, 300 mM sucrose, 100 mM NaCl, 0.5 mM EGTA, 5 mM MgCl $_2$, 50 mM NaF, 20 μ g/ml PMSF and 20 μ g/ml benzamidine, resuspended in 25 μ l of PTP assay buffer (25 mM imidazole, pH 7.5, 1 mg/ml BSA, 15 mM 2-mercaptoethanol) and used to assay the associated PTP activity as previously described [18]. PTP was assayed by measuring the release of [32 P]P $_i$ from 32 P-labelled myelin basic protein $\{(2-4) \times 10^6$ c.p.m./nmol [22]]. The PTP activity in each assay was calculated after subtracting the P $_i$ released from control immunocomplexes prepared with unrelated antibodies, and is given in units of fmol $\cdot$ min $^{-1}$ $\cdot$ mg $^{-1}$.

Peptide competition experiments

Met was immunoprecipitated from GTL-16 cells, and the immunocomplexes were washed and resuspended in 35 μ l of PTP assay buffer, as described above, and incubated in the presence of the indicated amounts of Met-derived peptides. Either the 8-mer phosphopeptides (Figure 1) or the wild-type or mutated 9-mer peptides (Table 2) were used. After shaking for 20 min at 4 $^{\circ}$ C, the immunocomplexes were washed once in PTP assay buffer to remove the unbound peptides and used to assay the associated PTP activity, as described above. The activity in each assay was calculated after subtracting the P $_i$ released from control immunocomplexes prepared with unrelated antibodies and incubated with the appropriate peptide.

Binding of PTP activity using the Met-derived peptides

Portions of 4 nmol of the biotinylated derivatives of the phospho- or dephospho-peptide described in the Materials section (residues 998–1007) were bound to 10 μ l of UltraLink NeutrAvidin beads according to the manufacturer's instructions. The peptide beads were then mixed with 2.5–3.5 mg of GTL-16 cell extract, incubated on a shaker at 4 $^{\circ}$ C for 90 min, washed and used to assay the associated PTP activity as described above for the immunoprecipitates. Activity was calculated after subtracting the P $_i$ released from control beads prepared without peptide or with an unrelated peptide.

Peptide affinity chromatography

A peptide affinity column was prepared using 0.5 ml of the UltraLink Immobilized NeutrAvidin support and 360 nmol of the biotinylated phospho- or dephospho-peptide. After washing the column with the same buffer used to wash the immunocomplexes (see above) containing 0.1% Triton X-100 and 15 mM 2-mercaptoethanol (column buffer), at a flow rate of 15 ml/h, 100 mg of GTL-16 cell extract was applied and recirculated four times through the column, to allow PTP binding. This was followed by washing with column buffer and stepwise elution with buffers containing 0.2, 0.3 and 0.5 M NaCl (see Figure 2). Fractions of 0.5 ml were collected and used to assay the PTP activity [18,22] and protein [23]. Figure 2 does not show activity present in the volume that was recirculated through the column, a value 4–5 times the activity present in the wash fractions (1–12). One unit of PTP activity releases 1 nmol of [32 P]P $_i$ /min from 32 P-labelled myelin basic protein (see above).

Western blotting

Fractions obtained from the peptide affinity column were precipitated in the presence of 0.003 % (w/v) deoxycholate and 7 % (w/v) trichloroacetic acid, subjected to electrophoresis on SDS/PAGE (10 % gels) and transblotted on to Immobilon-P (according to the manufacturer's instructions). Immunodetection used anti-(mouse IgG)-peroxidase and the ECL system [24].

RESULTS

Direct interaction of a PTP activity with the juxtamembrane domain of Met

We have shown previously that a PTP activity co-immunoprecipitates with Met from several cell lines (GTL-16, A549, HT-29) [18]. In order to identify the region responsible for binding the PTP, we used various deletion mutants of the receptor β -subunit. These were expressed permanently in NIH-3T3 cells and were all active as tyrosine kinases. The mutant receptors were immunoprecipitated and used to assay the associated PTP. First we used Tpr-Met, a construct containing residues 1010–1390 of Met fused to the Tpr sequence [15], which lacks the extracellular and transmembrane domains and the first 54 N-terminal amino acids of the juxtamembrane domain. When the receptor was immunoprecipitated, no associated PTP activity was found (Table 1). We used also NIH-3T3 cells expressing full-length Met, which binds PTP, as positive control (Table 1) and non-transfected NIH-3T3 cells, that do not contain Met, as negative control (results not shown). The results indicate that the first 54 amino acids of the juxtamembrane region are responsible for PTP binding.

In order to further identify the sequence involved in PTP binding, we used two constructs in which the portions of the

Figure 1 Effects of three phosphopeptides derived from the juxtamembrane region of Met on Met-associated PTP activity

Met was immunoprecipitated from 2.5–3.0 mg of cell extract and the immunocomplexes were incubated in the presence of the indicated quantity (0.1, 1.0 or 10 μ M) of one of the phosphopeptides, containing P-Tyr¹⁰⁰³ (▲), P-Tyr⁹⁷¹ (●) or P-Tyr¹⁰²⁶ (■), or with an unrelated phosphopeptide (◆; see also the Materials and methods section). At the end of the incubation, the excess peptide was removed and the associated PTP activity was assayed. For each determination the activity ($\text{fmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$) was calculated after subtracting the P_i released from a control immunoprecipitate prepared with an unrelated antibody and incubated with the same amount of phosphopeptide. Results are means $\pm$ S.E.M. of three determinations.

Table 1 PTP activity associated with Met mutants

Depiction of Met β -subunit: open box, kinase domain; closed box, juxtamembrane domain, indicating the position of Tyr¹⁰⁰³ (Y); stippled box, Tpr-derived sequence. The receptor mutants were expressed either permanently in NIH-3T3 cells (full-length, Tpr-Met, cyto-1 and cyto-2) or transiently in COS-1 cells (Tpr-juxta-Met and Tpr-juxta-Met carrying the Y $\rightarrow$ F mutation at site 1003). The receptors were immunoprecipitated using an anti-C-terminus antibody (DQ-13) and the associated PTP activity was assayed. Each determination is the average of the PTP activities associated with two immunoprecipitates, prepared from 2.5–3.5 mg of cell extract. The activity was calculated after subtracting the P_i released from two control immunoprecipitates, prepared with an unrelated antibody. Values are means $\pm$ S.E.M., numbers of determinations are in parentheses.

Construct	PTP activity ($\text{fmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$)
Full-length	65 $\pm$ 16 (n=5)
Tpr-Met	0 (n=8)
cyto-1	30 $\pm$ 14 (n=3)
cyto-2	27 $\pm$ 11 (n=5)
Tpr-juxta-Met	56 $\pm$ 22 (n=8)
Tpr-juxta(Y $\rightarrow$ F)-Met	116 $\pm$ 31 (n=5)

juxtamembrane domains were longer than in Tpr-Met and which also lacked the extracellular and transmembrane domains. In the cyto-1 mutant (residues 957–1390) only one amino acid was absent, whereas in the cyto-2 mutant (residues 995–1390) the first 39 N-terminal amino acids of the juxtamembrane domain were missing [15]. Both of these proteins had associated PTP activity (Table 1). Together, the results indicated that the sequence required to associate with the PTP is localized between residues 995 and 1009.

A juxtamembrane-derived phosphopeptide containing Tyr¹⁰⁰³ influences Met-associated PTP activity

Since some PTPs bind to phosphorylated Tyr residues through their SH2 domains, we investigated the possibility that a tyrosine-phosphorylated site in the Met sequence could be involved in PTP binding. Of the three Tyr residues in the juxtamembrane domain (Tyr⁹⁷¹, Tyr¹⁰⁰³ and Tyr¹⁰²⁶) only Tyr¹⁰⁰³ is included in the region between residues 995 and 1009. To test the ability of this residue to influence the associated PTP activity, we immunoprecipitated Met from GTL-16 cells (the system that yields the highest amount of associated PTP) and investigated whether PTP activity was affected by the presence of an 8-mer phosphopeptide containing Tyr¹⁰⁰³ (residues 1003–1010). Phosphopeptides containing Tyr⁹⁷¹ (residues 971–978) or Tyr¹⁰²⁶ (residues 1026–1033) or an unrelated phosphopeptide (see the Materials and methods section) were used as controls. Figure 1 shows that, in the presence of the phosphopeptide containing Tyr¹⁰⁰³, most or all of the Met-associated PTP activity, depending on the phosphopeptide concentration, was lost. The decrease in PTP activity could be explained by competition between the phosphopeptide and Met for PTP binding. This further suggested the

Table 2 Effects of wild-type and mutated peptides derived from the juxtamembrane region of Met on Met-associated PTP activity

Met was immunoprecipitated from 2.5–3.0 mg of extract. The immunocomplexes were incubated with 1 μ M of peptide Glu-Ser-Val-Asp-Tyr-Arg-Ala-Thr-Phe (residues 999–1007 in the juxtamembrane region of Met; wild-type peptide) or of peptides in which one residue had been mutated as indicated. At the end of the incubation the excess peptide was removed and the associated PTP activity was assayed. For each determination the activity was calculated after subtracting the P_i released from a control immunoprecipitate (prepared with an unrelated antibody and incubated with the same peptide). Values are means $\pm$ S.E.M. ($n = 3$).

Peptide (1 μ M)	PTP activity (fmol $\cdot$ min $^{-1}$ $\cdot$ mg $^{-1}$)
Wild-type	60 $\pm$ 17
Glu ⁹⁹⁹ $\rightarrow$ Ala	196 $\pm$ 19
Asp ¹⁰⁰² $\rightarrow$ Ala	221 $\pm$ 35
Arg ¹⁰⁰⁴ $\rightarrow$ Ala	205 $\pm$ 44

involvement of the sequence comprising the peptide in PTP binding. Figure 1 also shows that incubation with the other three phosphopeptides affected PTP activity in a concentration-dependent manner. However, this seems to be a non-specific effect, since it also occurred with the unrelated phosphopeptide.

Phosphorylation of Tyr¹⁰⁰³ is not required for PTP binding

In order to investigate the requirement for phosphorylation at site Tyr¹⁰⁰³, we used two Tyr-juxta-Met constructs (see Table 1): one with Tyr at position 1003, and one carrying a mutation of Tyr¹⁰⁰³ into Phe (an amino acid that mimics a dephosphorylated Tyr). Both constructs were transiently expressed in COS-1 cells and were active as tyrosine kinases. PTP activity was found associated with both products, although it was higher in the case of the point mutation into Phe (Table 1). These experiments indicated that phosphorylation of Tyr¹⁰⁰³ was not required for PTP binding, although it did not prevent it.

To confirm that the presence of phosphorylated Tyr¹⁰⁰³ is not required to bind PTP we used the biotinylated derivative of a peptide containing Tyr¹⁰⁰³ (residues 998–1007 of Met; see the Materials and methods section), prepared in both phospho and dephospho forms. The peptides were linked to an avidin-containing bead (UltraLink Immobilized NeutrAvidin) and used to bind PTP from a GTL-16 cell extract. The activity associated with the peptide was 44 ± 23 fmol $\cdot$ min $^{-1}$ $\cdot$ mg $^{-1}$ for the phosphopeptide and 85 ± 16 fmol $\cdot$ min $^{-1}$ $\cdot$ mg $^{-1}$ for the dephosphopeptide ($n = 5$). Each determination is the average of the PTP activities associated with two peptide beads, prepared from 2.5–3.5 mg of cell extract. The activity was calculated after subtracting the P_i released from two control beads, prepared without peptide. The results indicated that both peptides were associated with PTP activity. The higher values obtained with the dephosphopeptide may indicate either the presence of more PTP or competition between the phosphopeptide and the phosphorylated substrate myelin basic protein. Altogether, the results allowed us to conclude that the sequence around the phosphorylated site is responsible for the binding of PTP, and confirmed that phosphorylation of the site at Tyr¹⁰⁰³ is not required.

Mutations in the peptide containing Tyr¹⁰⁰³ abrogate the ability of the peptide to influence Met-associated PTP activity

In order to investigate further the role of the sequence around Tyr¹⁰⁰³ in PTP association, we used three peptides derived from

Figure 2 Isolation of PTP-S by peptide affinity chromatography

A portion of 100 mg of GTL-16 cell extract were applied to a 0.5 ml affinity column prepared with the Met-derived biotinylated phosphopeptide comprising residues 998–1007 and UltraLink Immobilized NeutrAvidin, as described in the Materials and methods section. The flow rate was 15 ml/min, and 0.5 ml fractions were collected. After washing with buffer at 0.1 M NaCl and elution at the indicated NaCl concentrations (broken line), PTP activity (●) and protein (▲) were assayed. One unit of PTP activity releases 1 nmol of [32 P]P/min from 32 P-labelled myelin basic protein. The fractions were pooled as indicated by the bars (pools A–F) and the proteins were concentrated by precipitation with trichloroacetic acid. Inset: 15% of each pool was subjected to electrophoresis and transfer on to Immobilon-P. PTP-S (S) was detected by probing the blot with anti-PTP-S monoclonal antibodies, followed by peroxidase-conjugated anti-(mouse IgG) and ECL reagents. Molecular mass markers: BSA (68 kDa), ovalbumin (43 kDa) and carbonic anhydrase (29 kDa).

the Met sequence comprising residues 999–1007 but carrying a mutation of Glu⁹⁹⁹, Asp¹⁰⁰² or Arg¹⁰⁰⁴ into Ala. The non-mutated (wild-type) peptide was used as positive control, since this was expected to decrease the Met-associated PTP activity. Table 2 shows that incubation with each of the mutated peptides preserved PTP activity associated with Met at much higher levels than incubation with the wild-type peptide. These results indicate that at least three residues in the sequence around Tyr¹⁰⁰³ are essential for PTP binding.

Identification of PTP-S binding to the Met juxtamembrane region

Identification of the sequence responsible for associating with PTP allowed us to design an affinity column in which the biotinylated derivative of peptide 998–1007 was bound to the UltraLink NeutrAvidin support. Figure 2 shows the profile of the column prepared with the phosphopeptide; the same results were obtained with a column prepared with the dephosphopeptide (not shown). The PTP activity bound by the column eluted in two peaks, at 0.2 M NaCl (peak at fraction 20) and 0.3 M NaCl (peak at fraction 26), and partially overlapped the last part of the wash peak (containing unbound PTP). The eluted fractions were pooled as indicated by the bars (pools A–F) and the proteins in the pools were concentrated by precipitation with trichloroacetic acid. Aliquots of each pool were subjected to

electrophoresis and transblotting, and the blots were probed with antibodies directed against various PTPs (including the human homologue of LAR, PTP δ , PTP- α and TC-PTP). The only probe that gave a positive result was that with anti-PTP-S antibodies (inset of Figure 2). This demonstrated the presence of PTP-S in pools C, D and E (corresponding to the first and second elution peaks), and, to a lesser degree, in pool A (corresponding to unbound PTP). PTP-S had a molecular mass of 43 kDa, as expected [21], did not correspond to any prominent band on a silver-stained gel of the pools (results not shown), and its identification required the specific antibodies. PTP-S was also immunoprecipitated directly from GTL-16 cell extracts and was detected by immunoblotting as a poorly immunoreactive band (results not shown). This confirmed that affinity chromatography was required in order to identify the Met-interacting PTP.

DISCUSSION

In this paper we identify the Met-associated phosphatase described previously as PTP-S. In Met immunocomplexes, such activity was very low, and this may explain the failure to detect the PTP protein even in immunocomplexes obtained from several milligrams of cell extract. Only the identification of the juxtamembrane sequence that binds the PTP allowed us to design a peptide affinity column, which led to the identification of PTP-S. The PTP activity retained by the affinity column represented less than 0.1% of the activity applied. This value is close to that calculated for the PTP activity associated with the Met immunocomplexes [18].

The findings that, *in vivo*, PTP also associated with receptors carrying the Tyr¹⁰⁰³ → Phe mutation and that, *in vitro*, peptide phosphorylation was not required to bind the PTP excluded the involvement of SH2 domains and pointed to a role for the peptide sequence. This specific sequence in the Met receptor might represent a docking or interaction site for the PTP or, alternatively, this site might be a specific PTP substrate. Acidic residues N-terminal to phosphotyrosine (such as Asp at -1 and Glu at -4 relative to Tyr¹⁰⁰³) have been reported in peptides that are substrates for soluble PTPs (reviewed in [25]). Mutation of these acidic sites, as well as of the Arg residue at the +1 position relative to Tyr¹⁰⁰³, influenced the ability of the peptide to compete with the receptor for PTP binding. This is a clear indication that the binding of the PTP to this site is highly specific.

The juxtamembrane domain of the Met receptor plays a negative role in the regulation of several biological activities. In particular, mutation of Tyr¹⁰⁰³ or of the surrounding residues (Thr at +3, Arg at +1, Asp at -1 and Val at -2 relative to Tyr¹⁰⁰³) resulted in increases in Met-mediated biological effects [26]. This suggests that this site could negatively modulate Met signalling. The discovery of a PTP that binds to this site supports the hypothesis of a regulatory role for Tyr¹⁰⁰³.

In our previous work [18], we showed that Met phosphorylation and activation were accompanied by increase in the associated PTP activity in an HGF-dose-dependent and time-dependent fashion. This indicated either that a PTP was recruited by the activated receptor or that a PTP already present underwent activation. PTP activation could be due to phosphorylation, as reported in the case of several PTPs (e.g. see [1]). The identification of the PTP that binds to a specific juxtamembrane region of Met will help to clarify the mechanism of PTP activation following stimulation by HGF.

PTP-S is a soluble PTP that was first cloned from a rat spleen library [27] and was subsequently identified in the cytoplasm and

nucleus in several cell types, including fibroblasts, liver and other epithelial cells [21]. Its sequence is closely related to that of TC-PTP, although it is not the rat homologue of human TC-PTP, and mouse and human variants of PTP-S have been found [28]. Two major PTP-S isoforms have been described: PTP-S4, which is cytosolic, and PTP-S2, which localizes to the nucleus and associates with chromatin [28]. Stimulation of fibroblasts and liver cells with mitogens and liver regeneration resulted in an increase in the level of PTP-S mRNA, raising the possibility of a role for PTP-S in cell proliferation [28]. The physiological substrates of PTP-S are unknown. The hypothesis that one substrate might be the HGF receptor Met will have to be tested.

In summary, the present work has addressed the issue of where the PTP associates with Met, the sequence requirements and which PTP is involved. This information will be useful in clarifying the role of the PTP in Met regulation.

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