Identification of Tyrosine Phosphatases That Dephosphorylate the Insulin Receptor

A BRUTE FORCE APPROACH BASED ON “SUBSTRATE-TRAPPING” MUTANTS*

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Many pharmacologically important receptors, including all cytokine receptors, signal via tyrosine (auto) phosphorylation, followed by resetting to their original state through the action of protein tyrosine phosphatases (PTPs). Establishing the specificity of PTPs for receptor substrates is critical both for understanding how signaling is regulated and for the development of specific PTP inhibitors that act as ligand mimetics. We have set up a systematic approach for finding PTPs that are specific for a receptor and have validated this approach with the insulin receptor kinase. We have tested nearly all known human PTPs (45) in a membrane binding assay, using “substrate-trapping” PTP mutants. These results, combined with secondary dephosphorylation tests, confirm and extend earlier findings that PTP-1b and T-cell PTP are physiological enzymes for the insulin receptor kinase. We demonstrate that this approach can rapidly reduce the number of PTPs that have a particular receptor or other phosphoprotein as their substrate.

Many cellular receptors signal via tyrosine phosphorylation (1, 2). The tyrosine kinases required for this activity are often recruited upon ligand binding, as in the Jak-Stat pathways utilized by cytokine receptors (growth hormone, interleukin-10, leptin, leukemia inhibitory factor, tumor necrosis factor, and interferon receptors). Alternatively, receptors themselves have kinase activity, like epidermal growth factor, brain-derived neurotrophic factor, glial cell line-derived neurotrophic factor, and insulin receptors. In either case, the receptors are returned to their original state through the activity of protein-tyrosine phosphatases (PTPs). Although nearly 50 distinct mammalian PTPs have now been identified (3), assignment of PTPs to receptors and other tyrosine phosphorylated substrates has been slow (4, 5). In theory, a pharmaceutical inhibitor of the PTP that is specific for a receptor would act as a receptor agonist. This concept is well illustrated in type II diabetes, where generic PTP inhibitors such as vanadyl salts have been known for over a hundred years to restore insulin sensitivity (cited in Ref. 6). In addition, a PTP knock-out mouse was recently shown to have increased insulin sensitivity (7). Because it is easier to develop drugs that block an enzyme than it is to find ligand agonists and because many of the ligands involved (growth hormone, leptin, insulin, and β-interferon) have therapeutic value, there is considerable interest in identifying specific PTPs for these receptors.

We have undertaken a systematic approach to PTP substrate identification that is based on “substrate-trapping” mutants of PTPs (5, 8–16). The most effective of these mutants have a Asp → Ala mutation about amino acids N-terminal of the catalytic HCSAG motif (3, 5, 15). These mutants, like their wild type counterparts, form an enzyme-substrate intermediate involving the conserved PTP cysteine and the tyrosine phosphate of the substrate but are consequently unable to release the dephosphorylated substrate. This approach has been used previously to identify intracellular substrates for PTPs (10, 12, 16). We have cloned the catalytic domains of the (nearly) full set of known human PTPs and expressed them either as wild type or as trapping mutants in glutathione S-transferase (GST) fusion proteins in bacteria. We have used these PTPs to test their affinity for the autophosphorylated insulin receptor kinase (IRK). Positive PTPs were validated in secondary assays for dephosphorylation of full-length IRK and of an IRK phosphopeptide that contained the tyrosines that are autophosphorylated in the IRK.

EXPERIMENTAL PROCEDURES

PTP cDNA Cloning—Oligonucleotides were designed for amplifying the ~1-kilobase pair catalytic domains of PTPs listed previously (3), plus Lyp-1 (GenBank™ accession number AF001846), EFMA2/Laforin (accession number AJ130763), and Esp (accession number MMU36488). The sequence of the PTPs corresponded to the entire similarity domain (Ref. 3; available on request). The oligonucleotides were designed to give the polymerase chain reaction (PCR) products a 5′ EcoRI site (or a MunI site, if the fragment already had an EcoRI site), and a 3′ NolI site, preceded by an artificial stop codon. The oligonucleotides were used in a PCR, using proofreading polymerases and various templates. If available, template was plasmid DNA from expressed sequence tag containing bacteria (Research Genetics). If these could not be used, DNA from various λ cDNA libraries was used, or reverse-transcribed human RNA (CLONTECH). In the latter two cases, nested PCR was performed. The tissue source for RNA or libraries was on the basis of where the PTPs were reportedly expressed or what source their expressed sequence tags, listed in public data bases, came from. The Asp → Ala point mutations (3) were introduced using specific oligonucleotides and PCR. The fragments were cloned in-frame into the pGEX4T3 (Amersham Pharmacia Biotech) EcoRI-NolI sites.

Expression of GST-PTP Protein—The plasmids were transformed into BL21 DE3 (Stratagene). A 0.25-ml overnight culture was expanded at 37 °C in 25 ml of LB until A600 0.5, induced in 0.2 mM isopropyl-1-thio-β-D-galactopyranoside and incubated a further 3 h at 30 °C. The bacteria were centrifuged, washed in PBS, and taken up in 1.5 ml of buffer (50 mM Tris-Cl, pH 8, 5 mM EDTA, 0.1% Triton X-100, 150 mM NaCl, 0.2 mg/ml lysozyme), followed by three sonications and centrifugation. The supernatant was incubated with 100 μl of glutathione-

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† The abbreviations used are: PTP, protein-tyrosine phosphatase; GST, glutathione S-transferase; IRK, insulin receptor kinase; PCR, polymerase chain reaction; PBS, phosphate-buffered saline; TC-PTP, T-cell PTP; pNPP, para-nitrophenylphosphate.
Sepharose (Amersham Pharmacia Biotech) beads. The beads were recovered by centrifugation at 2,000 rpm (HB4, Sorvall) and washed four times with 5 ml of PBST (PBS plus 1% Triton X-100, 2 mM EDTA, 5 mM benzamidine, 5 mM dithiothreitol, 5% glycerol), followed by two GST-PTP elutions of 100 μl glutathione solution (50 mM Tris, pH 8, 10 mM glutathione). The protein concentration was measured (Protein Assay Reagent, Bio-Rad), and the samples were stored in 20% glycerol at −20 °C.

**Phosphatase Activity Assay**—1 μg of GST-PTP was dissolved in 150 μl of buffer (50 mM Tris, pH 6.8, 2 mM dithiothreitol) and incubated at room temperature in a 96-well microtiter plate in 20 mM pNPP (Sigma P2525, 104 Phosphatase substrate number 104-100). The plates were read at 405. The wells were scored for the minimal time interval needed to reach an A405 of 0.2; >24 h is scored as 0, <24 h as 1, <4 h as 2, <1 h as 3, <15 min as 4, <5 min as 5, and <2 min as 6.

**IRK Production and Autophosphorylation**—Human IRK protein (cytoplasmic domain, amino acids D1002-end) was produced in baculovirus-infected Sf9 cells. Briefly, a bgH-1psrl cDNA fragment encoding the kinase domain was cloned into a derivative of pAc573 (17). In this vector the polyhedrin ATG start codon was mutated into ATG so that translation would start downstream, at the ATG preceding the IRK GAT-codon, which is the first aspartic acid after the transmembrane domain of the receptor. The construct was co-transfected with wild type virus into Sf9 cells, and recombinant virus was identified following several rounds of dot-blot hybridization in serial dilutions. For large scale production, Sf9 cultures were incubated at 1 ng/ml in 4-liter tanks at 27 °C in TC100 at 50% O2 saturation. Inoculated GST-PTP protein was incubated at 1 ng/ml for 90 min at 30 °C in 40 mM Tris, pH 7, 50 mM NaCl, 1 mM EDTA. The reaction was stopped solution contained 0.1 M Na3VO4 (Sigma S-6508) and 0.3 M H2O2. A 104 Phosphatase substrate number 104-100). The plates were read at 405. The wells were scored for the minimal time interval needed to reach an A405 of 0.2; >24 h is scored as 0, <24 h as 1, <4 h as 2, <1 h as 3, <15 min as 4, <5 min as 5, and <2 min as 6.

**Dot-Blot Trapping**—Nitrocellulose membrane (BA38 0.2 μm, Schleicher & Schuell number 401388) was fitted in a 96-well dot-blot apparatus (Bio-Rad 170–6545). IRK (0.5 μg in 50 μl of PBS) was incubated for 1 h in each well, followed by 1 h of blocking in 200 μl of washing buffer (PBS, 0.2% Tween-20) plus 5% nonfat milk powder. The GST-PTP protein was incubated at 1 ng/μl (100 μl) for 1 h at 4 °C, followed by washing of the wells. The membrane was taken out of the dot-blot apparatus and washed and then blocked in 10 ml of washing buffer (PBS, 0.05% Tween-20) plus 5% nonfat milk powder. The blot was washed and incubated for 1 h at room temperature followed by washing and incubation with goat anti-mouse Ig-horseradish peroxidase (1:1, Dako P0447). Detection was by chemoluminescence (ECL kit, Amersham Pharmacia Biotech RPN 2106).

**Dephosphorylation Assay of IRK**—GST-PTP (wild type) proteins were incubated at 1 ng in 10 μl with 20 ng of nonphosphorylated IRK in 40 mM Tris, pH 7, 50 mM NaCl, 1 mM EDTA. The reaction was stopped with 0.4 mM (final) paranovate after 0.5, 2, 15, or 30 min. The stop solution contained 0.1 mM Na3VO4 (Sigma S-6508) and 0.3 mM H2O2. The samples were spotted on membrane (BA38 0.2 μm Schleicher & Schuell number 401388), and the blot was blocked in 10 ml of washing buffer (PBS, 0.2% Tween-20) plus 5% nonfat milk powder. The blot was washed and incubated for 1 h at room temperature with anti-phosphotyrosine antibody (1:1500, clone 4G10 mouse monoclonal IgG2b washed and incubated for 1 h at room temperature with anti-phospho-buffer (PBS, 0.2% Tween-20) plus 5% nonfat milk powder. The blot was washed and incubated for 1 h at room temperature followed by washing and incubation with goat anti-mouse Ig-horseradish peroxidase (1:1, 500, Dako P0447). Detection was by chemoluminescence (ECL kit, Amersham Pharmacia Biotech RPN 2106).

**SDS-Polyacrylamide Gel Electrophoresis and Western Blot Detection**—Gels (Novex, 4–20%) were run according to the manufacturer's instructions and protein was transferred to polyvinylidene difluoride membrane using a dry-blot (Bio-Rad) transfer apparatus. Incubation with anti-tyrosine phosphate antibody was as described above.

**IRK Peptide Dephosphorylation Assay**—The wild type GST-PTPs (1.5 ng) were incubated with IRK phosphopeptide (GMTRDIY(FP)O2)-ETTLPST-1111) (Peptides International) or histone H3 (50 μM) in 40 mM HEPES, pH 7.2, 1 mM EDTA, 1 mM dithiothreitol, 0.05% Nonidet P-40, 0.2 mM peptide) in 96-well microtiter plates (Nunc). After 30 min of incubation at room temperature, 100 μl of Malachite Green Reagent (BioMol) was added, and A500 was determined on a Labsystems Multiscan Plate Reader. Activities were scored as − for an A500 < 0.10, + for an A500 = 0.2, ++ for an A500 = 0.32, and +++ for an A500 < 0.5.

**RESULTS**

**PTP Cloning**—The basis for cloning the PTP set was a recent compilation of unique, human PTP catalytic domains (3). The ~300 amino acids encoding cDNAs were cloned in-frame in a prokaryotic GST expression vector either as wild type PTP or with the substrate-trapping Asp → Ala mutation, and GST fusion proteins were produced and purified. Table I lists the PTPs, their bacterial expression levels and activities as measured with pNPP, a nonselective PTP substrate. A list with synonyms for the PTPs used can be found elsewhere (3). The last column of Table I lists conserved amino acid sequences found around the two main PTP catalytic centers. The “dual specificity” PTPs, those that also dephosphorylate serine and threonine phosphates (19), are less well conserved, with a list of nonselective PTP substrates. IRK was chosen as prototype substrate because it could autophosphorylate in vitro upon incubation with ATP (the protein produced in insect cells was nonphosphorylated). In addi-
tion, some PTPs have already been described that dephosphorylate the insulin receptor (21, 22), which allowed us to validate our approach. Early attempts to design a binding assay with mutant PTP and substrate with GST-PTPs immobilized on glutathione-agarose beads were unsuccessful because of non-specific binding of protein to the beads (data not shown). We then set up an assay whereby IRK was immobilized on a nitrocellulose membrane that was sandwiched in a 96-well blot apparatus. After blocking the membrane, each well was incubated with a different GST-PTP trapping mutant, and the whole membrane was incubated with an anti-GST antibody to detect PTPs that had bound to the receptor. As shown in Fig. 1, only a few out of the large panel of PTPs that were tested reacted with phosho-IRK, namely TC-PTP, PTP-1b, PTP-γ, and Sap-1. Although the latter two PTPs produced a lower signal, the assay gave a remarkably clear-cut readout. Modifying the concentration of GST-PTPs or the IRK on the membrane did not produce a different positive PTP set (data not shown). Well 25 (Fig. 1) contained GST protein that was directly bound to the membrane (positive control). The IRK samples in the Ctrl box were not autophosphorylated, indicating that binding of positive GST-PTPs to phosho-IRK depended on the latter containing phosphorylated tyrosine. Well 24 was incubated with GST fused with wild type Sap-1, showing that binding depended on the PTPs having a trapping mutation. The results shown in Fig. 1d show that PTP-1b and TC-PTP only bind as mutants. When a second set of PTPs was tested (Fig. 1a, lower panel), the PTPs that had tested positive earlier were retested, and these were found to bind reproducibly. The signals on the film were quantified and are shown in Fig. 1b. The strongest signal was obtained by PTP-1b, followed by TC-PTP, PTP-γ, and Sap-1. As shown in Fig. 1c, the IRK preparations that were used did not contain any other phosphorylated proteins that could potentially obscure the trapping results.

**Dephosphorylation of IRK by Wild Type PTPs**—To test whether the wild type version of PTPs that had tested positive in the protein-interaction assay was capable of dephosphorylating IRK, GST-PTP (wild type) was incubated with IRK for variable time intervals. To stop the reaction, we found that pervanadate (23) was more efficient than iodoacetate (24). Samples were spotted on nitrocellulose membrane, and the IRK phosphorylation state was examined using an anti-tyrosine phosphate antibody (Fig. 2). Interestingly, all PTPs that were tested could completely dephosphorylate the receptor, given sufficient time, including a negative control (PTP-PEST) that failed to bind IRK as a trapping mutant. However, the speed of conversion was different, with TC-PTP, PTP-1b, and Sap-1 displaying the fastest conversion. This type of result is similar to an earlier study that identified a substrate for PTP-PEST: the mutant PTP extracted a single substrate (p130(wt)), but dephosphorylation of a mixture of cellular phosphoproteins showed only a quantitative difference between activity on p130(wt) and other substrates (12). Thus, although this enzymatic assay confirmed the specificity of PTPs that have IRK as their substrate, results were much less straightforward than those that were obtained from the nitrocellulose membrane binding assay.

**Dephosphorylation of IRK Phosphopeptide by Wild Type PTPs**—Finally, we have tested the set of PTPs for their ability to dephosphorylate a phosphopeptide corresponding to the IRK autophosphorylation site. Like in the IGF-1 receptor, three tyrosines that are substrates for autophosphorylation are clustered in the IRK (25), and we designed a peptide that carried all three of these (GMTRDIYETDYYRKGKKG). Dephosphorylation of the triphosphopeptide by each PTP was monitored using a sensitive colorimetric assay for free phosphate (26), and the results are listed in Table II. TC-PTP, Sap-1, and PTP-1b all tested positive, but PTP-γ showed no activity, although the same GST-PTP preparation could efficiently convert pNPP (Table I). Interestingly, many other PTPs showed activity, namely DEP-1, GLEFP-1, LAR, PTP-β, -κ, -μ, and SHP-1. The PTPs that best dephosphorylate the phosphopeptide are not simply those that have the highest activity on pNPP (Table I), indicating that the peptide has higher PTP selectivity.
The phosphorylation state was determined using an anti-tyrosine phosphatase antibody. The reaction was stopped after different time intervals, the samples were spotted on membrane, and the IRK substrate trapping closely mimics physiological dephosphorylation. The binding on filters was inhibited by pervanadate and depended on the phosphatase being mutated and the substrate being phosphorylated. IRK substrate as bound on the nitrocellulose membrane had (at least partially) maintained correct folding, because incubation of nonphosphorylated IRK resulted in autophosphorylation (data not shown). The membrane binding assay also appeared to be robust in the sense that the set of PTPs testing positive did not depend on incubation time, as in the dephosphorylation assay (Fig. 2). Surprisingly, GST-PTP-γ was reproducibly positive in the membrane binding assay, yet the wild type protein failed to efficiently dephosphorylate IRK. The wild type GST-PTP-γ protein preparation had activity on nNPP and peptide substrates (Tables I and II). A possible explanation for this discrepancy is that three tyrosines are involved in IRK autophosphorylation. It is possible that PTP-γ recognizes and dephosphorylates only one or two of these. That might explain why complete dephosphorylation of the IRK is slow, whereas release of free phosphate from the IRK-peptide is efficient. Among the three PTPs that are positive in all three assays, PTP-1b and TC-PTP are structurally very similar (3). TC-PTP is abundantly and widely expressed, but homozygous mice mutated in this gene die soon after birth (34), so that the function of this PTP is as yet unresolved. Sap1 has very limited tissue expression that does unambiguously pick up in computer-assisted similarity searches. In the “PTP screen” described here we have chosen to focus on the catalytic domains of PTPs, because these domains are thought to be sufficient for substrate specificity (27), like kinase domains (28). On the substrate side we have selected the insulin receptor kinase, because it autophosphorylates and may therefore be considered as a physiological PTP substrate. In addition, IRK dephosphorylation has been intensively studied in the past, and those earlier results could serve as a yardstick for our approach. The major candidate PTPs previously implicated in IRK dephosphorylation are PTP-1b and LAR (29–31). Of these, PTP-1b is well established, because a mouse mutated in PTP-1b displayed highly increased insulin sensitivity (7). By contrast, LAR is expressed only at low levels in insulin-responsive tissues (31), and LAR knock-out phenotypes have been ambiguous (32, 33).

What we have found in our survey is that PTP specificity increased with substrates that were more physiologically relevant. Thus, nearly all PTPs hydrolyzed pNPP, a tyrosine-phosphate mimic; a subset dephosphorylated an insulin-like peptide and only four (PTP-1b, TC-PTP, PTP-γ, and Sap1) trapped the entire intracellular IRK. Our results confirm that mutant PTP substrate trapping closely mimics physiological dephosphorylation. The binding on filters was inhibited by pervanadate and depended on the phosphatase being mutated and the substrate being phosphorylated. IRK substrate as bound on the nitrocellulose membrane had (at least partially) maintained correct folding, because incubation of nonphosphorylated IRK immobilized on a membrane in suitable ATP-containing buffer resulted in autophosphorylation (data not shown). The membrane binding assay also appeared to be robust in the sense that the set of PTPs testing positive did not depend on incubation time, as in the dephosphorylation assay (Fig. 2). Surprisingly, GST-PTP-γ was reproducibly positive in the membrane binding assay, yet the wild type protein failed to efficiently dephosphorylate IRK. The wild type GST-PTP-γ protein preparation had activity on pNPP and peptide substrates (Tables I and II). A possible explanation for this discrepancy is that three tyrosines are involved in IRK autophosphorylation. It is possible that PTP-γ recognizes and dephosphorylates only one or two of these. That might explain why complete dephosphorylation of the IRK is slow, whereas release of free phosphate from the IRK-peptide is efficient. Among the three PTPs that are positive in all three assays, PTP-1b and TC-PTP are structurally very similar (3). TC-PTP is abundantly and widely expressed, but homozygous mice mutated in this gene die soon after birth (34), so that the function of this PTP is as yet unresolved. Sap1 has very limited tissue expression that does not coincide with IRK expression (35, 36); its involvement in IRK regulation is therefore doubtful. Interestingly, the IRK phosphopeptide that we have used is from a short sequence that is perfectly shared between IRK and insulin-like growth factor 1 receptor. It is therefore likely that among the set of PTPs that had tested positive in Table II are some that (also) have autophosphorylated insulin-like growth factor 1 receptor as their substrate.

Overall, we conclude that the combination of our membrane binding assay and activity tests, as applied to a large panel of PTPs, is a reliable first step in identifying potential substrates. The resulting small PTP set has then to be further evaluated in terms of intracellular localization of the PTPs (37), their tissue distribution, the effect of overexpression, and, ideally, gene inhibition using antisense or knock-out approaches. Shortening the list of PTP candidates before initiating these elaborate studies by a systematic approach as presented in this work should significantly shorten the path to PTP substrate assignments.
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