Activation of the SH2-containing Protein Tyrosine Phosphatase, SH-PTP2, by Phosphotyrosine-containing Peptides Derived from Insulin Receptor Substrate-1*

(Received for publication, December 20, 1993, and in revised form, February 28, 1994)

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The cytoplasmic insulin receptor substrate-1 (IRS-1), which is multiply phosphorylated in vivo on tyrosine residues, is a known binding protein for the tandem src homology 2 (SH2) domain-containing protein tyrosine phosphatase, SH-PTP2. Eleven phosphotyrosyl (pY) peptides from IRS-1 were screened for allosteric activation of SH-PTP2 phosphatase activity toward phosphorylated, reduced, carboxamidoethylated, and maleylated lysozyme. Peptides IRS-1pY895, IRS-1pY1172, and IRS-1pY1222 showed up to 50-fold acceleration of dephosphatase, SH-PTP2. Limited tryptic cleavage within the C terminus results in 27-fold activation of protein tyrosine phosphatase activity. The activated tryptic fragment cannot be further activated by pY peptide binding to the SH2 domains indicating that autoregulatory functions of the SH2 domains are dependent on the C-terminal region. These data suggest that multiple levels for control of SH-PTP2 enzymatic activity may exist in vitro and in vivo.

Many polypeptide growth factors signal via receptors with intrinsic protein tyrosine kinase activity (1). In recent years, much progress has been made in defining early events in receptor protein tyrosine kinase signaling (2, 3). Upon ligand addition, receptor protein tyrosine kinases dimerize, are enzymatically activated, and "trans-phosphorylate" at multiple sites (1) in their cytoplasmic domains. These tyrosyl phosphorylation sites serve as docking points to recruit secondary signaling molecules, many of which contain src homology-2 (SH2) domains and are also receptor protein tyrosine kinase substrates (1). SH2 domains, first identified in src-family protein tyrosine kinases, are regions of approximately 100 amino acids which bind with high affinity (e.g. 10^7 to 10^8 M^-1) to specific phosphotyrosyl (pY) peptides (3-9). The specificities of SH2 domains for particular phosphorylation sequences have been demonstrated in vitro by direct assays using SH2 domain and pY peptides (6-8) and in vivo by the finding that different pY residues on receptor protein tyrosine kinases associate with distinct downstream SH2 domain-containing proteins (3-5).

The specificity of the cellular response to growth factors may, in part, be determined by the strength and spectrum of these intermolecular SH2 domain/pY site interactions.

Although a general paradigm is emerging for early receptor protein tyrosine kinase signaling events, the role of specific protein tyrosine phosphatases (PTPases) in these pathways has remained obscure. Recently, we (10, 11) and others (12-18) have molecularly cloned a hematopoietic cell-specific PTPase, SH-PTP1 (10), also known as PTP1C (12), HCP (13), SHP (14), and PTPN6 (15), as well as a ubiquitously expressed PTPase, SH-PTP2 (11), also known as Syt1 (16), PTP1D (17), and PTP2C (18). Both of these enzymes contain two SH2 domains that precede a single phosphatase domain and a C-terminal region of unclear function.

SH-PTP2 is highly similar to and is likely the mammalian homologue of Drosophila csw (19). The csw gene product potentiates the action of the Drosophila homologue of mammalian c-raf to positively transmit signals downstream of the torso receptor protein tyrosine kinase (19, 20). Taken together, these data suggest an important role for SH-PTP2 in receptor protein tyrosine kinase signal transduction.

SH-PTP2 becomes tyrosyl (16, 17, 21) and threonyl (21) phosphorylated in response to growth factor stimulation and binds...
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to the ligand-activated platelet-derived growth factor receptor β (PDGFR) and epidermal growth factor receptor (16, 17, 21). In addition to these transmembrane proteins, SH-PTP2 also binds in vitro (21–23) and in vivo (22) to the tyrosine-phosphorylated form of insulin receptor substrate-1 (IRS-1).

Our goal is to clarify the inter- and intramolecular mechanisms of regulation of SH-PTPase activity. We reported the purification of full-length recombinant human SH-PTP2 produced in Escherichia coli and the characterization of its enzymological properties (24). We noticed that SH-PTP2 exhibits non-Michaelis-Menten kinetics toward a pY peptide encompassing Tyr1006 of the human PDGFR (PDGFRpY1009), which we (25) and others (26) found to be the high affinity binding site for SH-PTP2 on the PDGFR. Furthermore, addition of the synthetic pY peptide PDGFRpY1009, but not other pY peptides based on the PDGFR, resulted in activation of SH-PTP2 enzymatic activity (25). We also reported that a glutathione S-transferase (GST) fusion protein of the N-terminal SH2 domain of SH-PTP2 (N-SH2) binds directly to the PDGFR in vitro (25). Out of seven autophosphorylation sites of PDGFR, this fusion protein binds with high affinity to only one pY peptide (PDGFRpY1009) and the data suggest that SH-PTP2 binds to PDGFR via its N-SH2 domain.

In the present study we assess the respective contributions of the N- and C-terminal SH2 domains of SH-PTP2 in the recognition of specific pY peptides from IRS-1 and their ability to activate the SH-PTP2 catalytic domain. IRS-1 is directly phosphorylated at many tyrosine sites by the insulin receptor in vitro (21–23) and in vivo (22, 23). We demonstrate here that three pY peptides derived from IRS-1 are capable of specific binding to SH-PTP2 SH2 domains and of evoking up to 50-fold catalytic activation of SH-PTP2. Occupancy of either SH2 domain is communicated to the PTpase active site. In addition to regulation via its SH2 domains, we define a region of the SH-PTP2 C-terminus that also has a regulatory function.

EXPERIMENTAL PROCEDURES

Materials—E. coli cells harboring an expression plasmid for a GST fusion protein of amino acids 101–326 of SH-PTP2 (GST-SH2–C) were reported previously (27).

Plasmid Construction—For pET-ΔC61, a 1.6-kb Ndel-EcoRI fragment encoding amino acid residues 1–530 of SH-PTP2 was isolated from plasmid pET-ShPT2P, which is an expression vector of pET-11a containing the entire SH-PTP2 cDNA (24). The C-terminal two amino acids (amino acids 531–532) were reintroduced using two oligonucleotide adapters: 5′-TGAAGAAGATTTGAC′ (14-mer) and 5′-TGACTCACCCTCTCTC′ (15-mer), to facilitate subcloning. The 14- and 15-mer adapters and the 1.6-kb Ndel-EcoRI adapters were ligated into Ndel-Sall-linearized plasmid PET-ShPT2P.

For pET-2N, a 360-bp XbaI-BstYI fragment including 39 bp upstream of the structural gene and encoding amino acid residues 1–106 of SH-PTP2 was isolated from plasmid pET-2N and ligated into NdeI-EcoRI-linearized plasmid PET-ShPT2P. The C-terminal three amino acids (amino acids 107–109) were reintroduced using two oligonucleotide adapters: 5′-GATTTCAACTCTCTGTA′ (16-mer) and 5′-GATCTCGAACTCCAGCT′ (16-mer), to facilitate subcloning. The 16-mer adapters and the 360-bp XbaI-BstYI fragment were ligated into XbaI-Sall-linearized pET-ShPT2P.

For pGEX-2N, a 600-bp NdeI-EcoRI fragment encoding amino acids 1–109 of SH-PTP2 and containing the T7 terminator region of pET-11a was isolated from plasmid pET-2N and ligated into NdeI-EcoRI-linearized plasmid pGEX-3Xb, which is modified from pGEX-3X (Pharmacia LKB Biotechnology Inc.) to contain an Ndel site in the polylinker sequence (6).

For pET-R32K, the cDNA fragment encoding amino acid residues 138–224 of SH-PTP2 was amplified by the PCR, using as template the GST-C-SH2 expression plasmid pGEX-2C, as primers: 5′-TGAAGAACGACGACGGCCTGTAACATGGGGG-3′ (20-mer) and 5′-GATCTCGACATAGTTTCTGACGTCACGTA′ (15-mer), to substitute restriction site for amplification. The PCR product was digested with NdeI and XbaI and the 150-bp fragment was ligated into NdeI-Sall-linearized plasmid pET-ShPT2P. The sequence of the PCR-generated fragment was confirmed by DNA sequencing using the T7 promoter primer, 5′-TAATACGACTCACTATAGGG-3′ (20-mer).

For pET-R318K, the cDNA fragment encoding amino acid residues 1–73 of SH-PTP2 with substitution of Arg to Lys from pET-R32K was ligated into NdeI-EcoRI-linearized plasmid pET-ShPT2P. The sequence of the PCR-generated fragment was confirmed by DNA sequencing using the PCR PCR primer, 5′-TGAAGAACGACGACGGCCTGTAACATGGGGG-3′ (20-mer) and 5′-GATCTCGACATAGTTTCTGACGTCACGTA′ (15-mer), to substitute restriction site for amplification. The PCR product was digested with NdeI and XbaI and the 150-bp fragment was ligated into NdeI-Sall-linearized plasmid pET-ShPT2P. The sequence of the PCR-generated fragment was confirmed by DNA sequencing using the T7 promoter primer, 5′-TAATACGACTCACTATAGGG-3′ (20-mer).

Expression and Purification of SH-2 Domain GST Fusion Proteins—E. coli strain DH5α transformed with pGEX-2N and pGEX-2N 32K, respectively, was grown to express the GST fusion proteins. The GST fusion proteins were expressed and purified as wild type SH-PTP2, 5, 74–593 of SH-PTP2 (GST-C-SH2) was sequenced (27). These data suggest that SH-PTP2 binds to the structural gene and encoding amino acid residues 1–32 of SH-PTP2 and including 90 bp upstream was amplified by the PCR using as template the human PDGFR (PDGFRpY1009), which was cloned into the T7 promoter primer, 5′-TAATACGACTCACTATAGGG-3′ (20-mer) and 5′-GATCTCGACATAGTTTCTGACGTCACGTA′ (15-mer), to substitute restriction site for amplification. The PCR product was digested with NdeI and XbaI and the 150-bp fragment was ligated into NdeI-Sall-linearized plasmid pET-ShPT2P. The sequence of the PCR-generated fragment was confirmed by DNA sequencing using the T7 promoter primer, 5′-TAATACGACTCACTATAGGG-3′ (20-mer).
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RESULTS

Rat and human IRS-1 have been cloned (37, 38) and show high identity in amino acid sequence, especially regarding the position of tyrosines and their flanking sequences. Rat IRS-1 has 34 Tyr residues (37) and human IRS-1 has 32 Tyr residues. The position of tyrosines and their flanking sequences. Rat IRS-1 and human IRS-1 have been cloned (37, 38) and show high identity in amino acid sequence, especially regarding the position of tyrosines and their flanking sequences. Rat IRS-1 has 34 Tyr residues (37) and human IRS-1 has 32 Tyr residues (38), and all 32 Tyr residues of human IRS-1 are conserved in rat IRS-1. At 27 of the 32 conserved Tyr sites, the sequences of the 3 amino acid residues immediately following each Tyr are identical; such pYXXX motifs frequently represent docking sequences for SH2 domains (31, 32, 39). In this study, we used 11 synthetic peptides corresponding to rat IRS-1 sequences, including the known phosphorylation sites (23), to determine the effects of SH2 occupancy on SH-PTP2 PTPase activity. At 80 pm concentration, three out of the 11 IRS-1 derived pY peptides (IRS-1pY895, IRS-1pY1172, and IRS-1pY1222) demonstrated activation of SH-PTP2 PTPase activity toward pY-RCM-lysozyme (Fig. 1).

We chose these three pY peptides for studies of the dose dependence of activation (Fig. 2). Two other pY peptides (IRS-1pY546 and IRS-1pY727) that did not activate served as negative controls. IRS-1pY1172 produced a biphasic activation profile, in which SH-PTP2 was activated 15-fold at lower concentration of pY peptide (ED50 of 5–10 pm) and 25-fold further activation (total of 40-fold activation) was observed at higher concentrations of pY peptide (ED50 of 200 pm). Under these conditions, IRS-1pY895 and IRS-1pY1222 demonstrated monophasic activation (ED50 of 200 pm). Interestingly, IRS-1pY1222 evoked higher maximal fold activation (60-fold) than IRS-1pY1172 or IRS-1pY895 (40-fold). IRS-1pY546 and IRS-1pY727 showed no significant effect within the concentration range tested.

It was unclear why IRS-1pY1172 showed biphasic activation; we suspected that occupancy of either the N- and C-SH2 domains by pY peptides might provide allosteric activation and that IRS-1pY1172 might have a different affinity for each SH2 domain. Thus, at lower concentrations, IRS-1pY1172 could bind first to one SH2 domain and show activation to some extent, whereas at higher concentrations, it would bind to the second SH2 domain, yielding further activation. By contrast IRS-1pY895 and IRS-1pY1222 might bind to only one SH2 domain or have comparable lower affinity for both SH2 domains. To test this hypothesis, we analyzed SH2 domain recognition of IRS-1 pY peptides in two ways: (i) by examining the effects of SH2 domain mutations on SH-PTP2 activity and (ii) by direct determination of the Kd between pY peptides and each SH2 domain of SH-PTP2.

SH2 Domain Mutants: Effects on Allosteric Activation—Structural studies have established that a specific Arg residue in the SH2 domains of src, abl, and lck stabilizes the negatively charged phosphotyrosine group of bound pY ligands (31, 32, 39, 40). An Arg to Lys mutation at this conserved residue leads to significantly weaker binding of pY peptides (41). The Arg to Lys mutations were made in SH-PTP2 in the N-SH2 domain at Arg32, in the C-SH2 domain at Arg138, and in both SH2 domains. The three mutant enzymes were expressed in E. coli, purified to homogeneity, and their kinetic properties were compared with the wild type enzyme.

The double Arg to Lys substitution mutant, R32K/R138K, showed no catalytic activation up to 100 pm IRS-1pY1172, whereas wild type SH-PTP2 showed 25–30-fold activation at 100 pm IRS-1pY1172 (Fig. 3), confirming that IRS-1pY1172
activates SH-PTP2 via its SH2 domain(s). Activation of R32K/R138K by IRS-1pY1172 at concentrations greater than 200 μM may result from partial affinity of the SH2 domains for pY peptides.

The single R138K substitution mutant in the C-SH2 domain showed PTPase domain activation with an ED₅₀ of 5–10 μM, indicating that the wild type N-SH2 domain in R138K was responsible for high affinity activation. In the R32K N-SH2 domain mutant, no activation by IRS-1pY1172 was detected at concentrations less than 100 μM. Along with the results on the double substitution mutant, these data indicate that the N-SH2 domain is responsible for high affinity binding and activation.

However, at higher concentrations of IRS-1pY1172, the R32K mutant could be allosterically activated. Interestingly, at these higher concentrations (>100 μM) R32K displayed a steep activation curve, with maximal activation at ~2 μM IRS-1pY1172, whereas R138K or R32KR138K showed a more shallow activation profile. These data suggest that the C-SH2 domain can be occupied at higher concentration of IRS-1pY1172 and activate the catalytic domain.

Direct Analysis of Kᵦ of SH-PTP2 SH2 Domains for pY Peptides by Surface Plasmon Resonance—Purified GST fusion proteins of the N- or C-SH2 domains of SH-PTP2 were used to measure the Kᵦ for binding to immobilized IRS-1pY895, IRS-1pY1172, or IRS-1pY1222, by surface plasmon resonance analysis (6, 7, 33) (Table I). Typical sensorgrams are shown in Fig. 4 for binding of GST-N-SH2 to IRS-1pY1172 (Fig. 4A) or GST-C-SH2 to IRS-1pY1222 (Fig. 4B). Kᵦ values were calculated from rate constants of association (kₐ) and of dissociation (kᵦ) or measured from response units at equilibrium (Rₑ) and compared by the peptide competition assay (6) in Table I. The measured affinities of the three pY peptides for both SH2 domains varied over a wide range.

Most notably, IRS-1pY1172 shows high affinity (14 nm) for the N-SH2 domain, some 240-fold higher than its affinity for the C-SH2 domain. These results are qualitatively consistent with the mutagenesis studies described above. The fusion protein containing the R32K mutation, GST-N-SH2R32K, showed about 50-fold lower affinity to the IRS-1pY1172 surface (640 versus 14 nm) than the wild type N-SH2 domain.

Although the IRS-1pY895 peptide shows almost 30-fold lower affinity for the N-SH2 domain (390 nm) than IRS-1pY1172, its Kᵦ for the C-SH2 domain (310 nm) is some 10-fold less than IRS-1pY1172 for this SH2 domain. In relative terms, the IRS-1pY895 preference between N- and C-SH2 domains is 1:1 versus 240:1 for IRS-1pY1172, thus explaining the monophasic activation curve seen for IRS-1pY895 activation of PTPase activity (Fig. 2).

The third IRS-1 pY peptide, IRS-1pY1222, shows 60-fold lower affinity for the N-SH2 domain (900 nm) than IRS-1pY1172, whereas its Kᵦ for the C-SH2 domain (110 nm) is some 30-fold less than IRS-1pY1172 for this SH2 domain. Compared to the wild-type IRS-1pY1172, IRS-1pY1222 displayed the converse specificity, with a 1:8 preference for N- versus C-SH2 domains. Given the relative magnitudes of the Kᵦ values of IRS-1pY1222 for the N-SH2 and C-SH2 domains and the ED₅₀ for activation of PTPase activity, it is likely that activation by IRS-1pY1222 arises via the C-SH2 domain, although it is possible that the 8-fold difference in Kᵦ is small enough that biphasic activation by IRS-1pY1222 appears to be monophasic.

Role of the C-terminal Region of SH-PTP2 in Regulation of Catalytic Activity—In addition to the two tandem SH2 domains upstream of the catalytic PTPase domain, SH-PTP2 and SHPTP1 have additional amino acids (69 and 81 amino acids, respectively), downstream of the catalytic core (10, 11). Activation of SH-PTP1 by C-terminal truncation, releasing 41 amino acids, was reported previously (42). We were therefore interested in determining the effect of the SH-PTP2 C terminus on PTP activity and its possible relationship to regulation via the SH2 domains.

Full-length, wild type SH-PTP2 was subjected to limited digestion with either trypsin or chymotrypsin. Chymotrypsin digestion yielded a stable fragment about 3.5 kDa smaller than the wild type enzyme whereas trypptic digestion yielded a stable fragment about 4.4 kDa shorter (Fig. 5A). N-terminal sequence analyses of trypsin- and chymotrypsin-treated SH-PTP2 revealed that both retain the original N-terminal sequence (data not shown). Thus, both proteases cleave within the C-terminal region. Inspection of the SH-PTP2 protein sequence reveals the potential cleavage sites noted in Fig. 5B. The trypptic fragment is approximately 1 kDa larger than the chymotryptic fragment (Fig. 5A), consistent with the suggested sites of cleavage (Fig. 5B); however, a dramatic difference in basal PTPase specific activity was observed. The chymotryptic fragment possessed the specific activity of the wild type enzyme, but the specific activity of the trypsin-treated fragment was elevated 27-fold, indicating a disruption of autoregulation by tryptic cleavage (Table II). When these two fragments were assayed for activation by IRS-1pY1172, the chymotryptic fragment was activated 18-fold, comparable to the 30-fold activation with full-length enzyme. However, there was no further effect of IRS-1pY1172 addition to the trypsin-treated fragment, (already activated 27-fold) suggesting that the trypsin-cleaved enzyme was already fully activated.

We also analyzed the enzymological properties of an SH-PTP2 C-terminal deletion mutant (ΔC61). As shown in Fig. 5A, the purified ΔC61 protein had the expected molecular mass,
Fig. 4. Typical sensorgrams for determination of \( K_d \) values for binding of IRS-1 pY peptides to GST-SH2 fusion proteins. Various concentrations of GST-N-SH2 or GST-C-SH2 were passed over an immobilized IRS-1pY1172 or IRS-1pY1222 sensor chip surface for 5 min at a flow rate of 5 \( \mu \)l/min. A, sensorgrams for IRS-1pY1172 binding to the indicated concentrations of GST-N-SH2 are shown. The region used to determine \( k_r \) was 250–300 s for GST-N-SH2 concentration of 25 nM, 210–250 s for 50 nM, 175–210 s for 100 nM, and 150–159 s for 300 nM. The region for \( k_d \) determination was 450–540 s. The \( R_u \) data point used to determine \( K_d \) was 447 s. B, binding of the indicated concentrations of GST-C-SH2 to IRS-1pY1222 are shown. The region used to determine \( k_r \) was 170–200 s for GST-C-SH2 concentration of 2 \( \mu \)M, 174–250 s for 4 \( \mu \)M, 165–250 s for 6 \( \mu \)M, 165–200 s for 8 \( \mu \)M, and 165–290 s for 10 \( \mu \)M. The region for \( k_d \) determination was 460–565 s.

apparently 5 kDa less than full-length enzyme. Surprisingly, the AC61 protein, which is only slightly smaller than the (activated) protein generated by tryptic cleavage, retained wild type basal specific activity and the capacity for marked activation (19-fold) by IRS-1pY1172.

Three points emerge. First, proteolysis by trypsin at a spe-
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**DISCUSSION**

PTPases are of interest both for their catalytic mechanism, involving a covalent S-phosphocysteinyl enzyme intermediate (43–46) and for their biological role in the regulation of signal transduction. The SH2 domain-containing PTPases, SH-PTP2, examined here, and SH-PTP1, are of particular enzymological interest because they contain three domains capable of recognizing pY proteins: two high affinity SH2 domains and the lower affinity catalytic domain, which possesses an irreversible hydrolytic function. In the evaluation of the possible biological roles of SH-PTP2, some attention to date has focused on assessing the binding selectivity and specificity of the two SH2 domains for pY peptides.

Songyang et al. (9) screened a pY peptide library with GST fusions of the N-SH2 or C-SH2 domains of SH-PTP2 and observed binding only to the GST-N-SH2 with a sequence preference of pY-I/V-X-V/I. More recently, based on the results of peptide competition assays, Case et al. (27) suggested the refinement pY-V/I/T-X-V/I/I for N-SH2 domain binding; that is, a preference for β-branched amino acids at the +1 and hydrophobic residues at the +3 positions. Previously, we did not isolate ligands to the C-SH2 domain, possibly due to the use of a shorter construct of GST-C-SH2 (9, 21). We have previously reported a functional assay to assess the occupancy of SH2 domains by pY ligand-coupled activation of full-length SH-PTP2. Using pY peptides from the PDGFR, we noted that PDGFRpY1009 (SVLpYTAYQPNE) stimulated catalytic activity, measured as release of 32P, from 32P-pY-RCM-lysozyme (25). This biochemical observation is likely to have important implications for SH-PTP2 function in vivo, since the PGDFR is a likely relevant in vivo partner of this PTPase (16, 17, 21, 22), and we (25) and others (26) have identified pY1009 as the high affinity site for SH-PTP2 binding to the activated PDGFR.

A second likely partner for SH-PTP2 in signal transduction cascades is the cytoplasmic substrate of the insulin receptor, IRS-1 (21–23). This protein is multiply phosphorylated in vivo (22, 23) as well as in vitro (21, 22) and has been shown to recruit such SH2-containing proteins as phosphatidylinositol 3-kinase (PI3K)(47, 48), GRB2 (49–51), and SH-PTP2 (22, 23). Recently, Sun et al. (23) used IRS-1 phosphorylated by the insulin receptor to map several IRS-1 tyrosine phosphorylation sites including Tyr80, Tyr=95, Tyr=39, Tyr=57, Tyr=1172, and Tyr=1222. These studies revealed that tryptic peptides containing pY808, pY895, and pY1172 bound to the SH2 domains of the p85 subunit of PI3K, GRB2, and SH-PTP2, respectively. Independently, Case et al. (27) measured the relative affinity of 13 IRS-1pY peptides for the N-SH2 domain of SH-PTP2 (IRS-1pY146, IRS-1pY460, IRS-1pY546, IRS-1pY608, IRS-1pY628, IRS-1pY658, IRS-1pY727, IRS-1pY895, IRS-1pY939, IRS-1pY987, IRS-1pY1010, IRS-1pY1172, and IRS-1pY1222) and found that IRS-1pY1172 bound most tightly, followed by IRS-1pY895.
peptides IRS-1pY895, IRS-1pY1172, and IRS-1pY1222 of the 11 studied showed stimulation of PTPase activity. This stimulation was dramatic, up to 40–60-fold increase of V_{max}, even more pronounced than the 10–15-fold effect seen with PDGF-FRAP1009 (25).

To further analyze the contributions of the individual SH2 domains of SH-PTP2 to allosteric activation, mutagenesis of the conserved arginines, Arg^{32} (N-SH2) and Arg^{138} (C-SH2) in either or both of the SH2 domains was undertaken. The data on the R32K/R138K double mutant are particularly compelling in that the allosteric activation of PTPase activity by IRS-1pY1172 is reduced by two orders of magnitude, proving that one or both SH2 domains mediate(s) SH-PTP2 activation. Moreover, these data, combined with our previous demonstration (25) that activation was dependent on the phosphorylation of the tyrosine-containing peptides, strongly suggests that activation is mediated via phosphotyrosine-dependent binding to the SH2 domains.

The R32K single mutant is ~50-fold less responsive than wild type, whereas the R138K mutant retains some high affinity activation capacity (~6-~8-fold). These results suggest that the N-SH2 domain is indeed the high affinity IRS-1pY1172-binding site and that occupancy of the N-SH2 domain alone (at low concentrations of IRS-1pY1172) is sufficient to evoke some level of SH-PTP2 activation. However, several lines of evidence also indicate that occupancy of the C-SH2 domain can also lead to allosteric activation.

IRS-1pY1172 shows a biphasic dose-dependence activation curve, with maximal activation occurring at millimolar concentrations of IRS-1pY1172. This observation is most easily explained by binding of this peptide to the N-SH2 at low concentrations yielding partial SH-PTP2 activation followed by C-SH2 domain occupancy at higher concentrations leading to full activation of the enzyme. Consistent with this notion, the R32K mutant, which lacks a functional N-SH2 domain, can be activated by IRS-1pY1172 but only at high concentrations of the peptide (maximal activation approached at 2 mM IRS-1pY1172). Likewise, the binding of both R32K and R138K have weakened ability to elicit full activation at higher IRS-1pY1172 concentrations implies that the C-SH2 domain functions in activation at higher concentration of IRS-1pY1172. The mutagenesis data indicate that both of the SH2 domains participate in allosteric activation of the PTPase domain. Moreover, from a biological standpoint, these data raise the possibility that upon interaction with different target proteins containing distinct pY peptide sequences different extents of PTPase activation may occur in vivo. Such differences could have important consequences for downstream signaling.

Direct determination by surface plasmon resonance of the affinity of the N-SH2 and C-SH2 domains for the three activating IRS-1 pY peptides revealed additional correlations. IRS-1pY1172 (pYIDDL) with a K_{d} of 14 nM is a high affinity ligand for the N-SH2 domain; some 50-fold loss in affinity is observed with the GST-N-SH2R32K mutant. The IRS-1pY895 sequence (pYIVN) is recognized 30-fold less well and the pYASI sequence of IRS-1pY1222 some 60-fold less well by the N-SH2 domain. Our results differ somewhat from those of Case et al. (27), who reported that IRS-1pY1172 binds 4–5-fold more tightly to the N-SH2 domain than IRS-1pY895 (compared with the 50-fold preference observed in this study; see Table I). This discrepancy is probably due to methodological differences between the two studies. In particular, it should be noted that the GST-N-SH2 domain fusion protein used by Case et al. (27) was refolded during purification. This process might lead to slight decreases in the binding ability of the fusion protein.

Analyses of the C-SH2 domain reveal data supporting its ability as an isolated domain to selectively bind pY sequences.

More importantly, the C-SH2 domain displays substantially different binding preferences relative to the N-SH2 domain. Both IRS-1pY895 and IRS-1pY1172 bind to the C-SH2 domain 10–30-fold more tightly than IRS-1pY1172 with K_{d} values of 310–110 nM, respectively. This reversal of selectivity indicates that the N-SH2 and C-SH2 domains of SH-PTP2 recognize distinct pY sequences.

Together with the SH2 domain mutant results, these data suggest that the N-SH2 domain is responsible for sensitive allosteric activation by IRS-1pY1172, whereas the C-SH2 domain is responsible for the 60-fold allosteric activation by IRS-1pY1172 and contributes to activation by IRS-1pY895. This gives rise to the prospect that recognition and dephosphorylation of a multiphosphorylated IRS-1 by SH-PTP2 could involve allosteric engagement of two distinct pY sequences (e.g. IRS-1pY1172 in the N-SH2 domain and IRS-1pY1222 in the C-SH2 domain, given the range of IRS-1 peptides examined in this study), while a third pY sequence (either on IRS-1 or a distinct substrate) undergoes allosterically activated hydrolysis in the catalytic site (see Fig. 6).

It should also be noted (Fig. 2) that maximal activation (at saturation) by IRS-1pY1222 (60-fold) is greater than that produced by IRS-1pY895 and IRS-1pY1172 (40-fold). This suggests that different peptides, when bound to the same SH2 domain, may result in subtle differences in SH2 structure which can then have important regulatory consequences.

Prior work on SH-PTP1 (PTP1C) indicated that proteolytic cleavage of 41 amino acids from its C-terminal region enhanced basal enzymatic activity 15-fold (42). We observed similar behavior with SH-PTP2 upon limited exposure to trypsin, in which a ~4.4-kDa fragment is cleaved from the C terminus (Fig. 5 and Table II) to yield a catalytic fragment with a 27-fold higher V_{max} than the full-length enzyme. Notably, this truncated, activated form of SH-PTP2 shows no further activation by pY ligand binding to either SH2 domain. However, both a slightly larger fragment of SH-PTP2 generated by limited chymotryptic digestion and a slightly smaller fragment (AC61) generated by recombinant DNA techniques have basal level PTPase activity that is fully activatable by pY ligand occupancy of either SH2 domain.

Similar results have been observed with SH-PTP1 using two C-terminal deletion mutants ΔC35 and ΔC60 that were constructed and purified to homogeneity. Although activating pY peptides have not yet been found for SH-PTP1, SH-PTP1ΔC35 shows 41-fold higher PTPase activity toward pY-RCM-ly-
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D. Pei, S. Sugimoto, and C. T. Walsh, unpublished results.

1 These data suggest a model in which, in the absence of pY peptides, either or both SH2 domains inhibit PTPase activity and that the C-terminal region participates in autoinhibition by SH2 domain(s).

2 Therefore, it is likely that a region of SH-PTP2 from Glu205 to the C-terminal end of the chymotryptic fragment (estimated to be between residues 546–552) has the capacity to modulate PTPase activity. To clarify the C-terminal modulation mechanism of PTPase activity, further studies of the SH-PTP2 C terminus are required.

3 Similar discrepancies between binding and activation have been observed in other studies. It has been reported that IRS-1 pY peptides (pYMXX) bind to the SH2 domains of the p85 subunit of PI3K (47, 48) and activate the lipid kinase (54, 55).

4 The ED$_{50}$ for kinase activation is also 500–15,000-fold higher for the C-terminal region than that of IRS-1 in vivo.4 Thus, in vivo phosphorylation and/or GR2B binding might also influence activity of the enzyme, in addition to constitutive phosphorylation at Ser and Thr (16, 17, 21, 52). Since SH-PTP2 becomes tyrosyl phosphorylated in response to PDGF stimulation, the selective activation by specific IRS-1 pY sequences along the covalent S-phosphocysteinyl enzyme intermediate.

5 It is possible that the observed pY peptide binding to the N-SH2 domain is mediated by pY peptide binding to the N-SH2 domain. However, these effects are transmitted to the active site remain to be resolved. Future studies will be directed toward elucidating the molecular details of both inter- and intramolecular regulation of SH-PTP2 activity. In particular, it will be important to clarify whether and how these regulatory mechanisms influence specific PTP catalysis such as formation or breakdown of the covalent S-phosphocysteinyll enzyme intermediate. In summary, our studies establish that both SH2 domains of SH-PTP2 function in allosteric activation, that the C-terminal region is auto regulated, and that activation via SH2 domains is related to autoregulation of activity by the C-terminal region. The selective activation by specific IRS-1 pY sequences along with known behavior of IRS-1 as a ligand of SH-PTP2 suggest this will be an interesting protein pair with which to assess SH-PTP2 function.

Acknowledgments—We thank R. J. Lechleider for E. coli cells expressing GST-C-SH2 and the Dana Farber Cancer Institute for N-terminal amino acid sequencing analysis and oligonucleotide synthesis. We thank I. A. Stolz and G. Payne for helpful discussions about the BIACore studies and M. Yamada for assistance with peptide synthesis.

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