from a precursor molecule by proteolytic cleavage, which raises the possibility of tissue-specific regulation through protease activation. The structural homology of betacellulin with members of the EGF family and the relatively low affinity of betacellulin for the classical EGF receptor suggests that betacellulin might act through another receptor. The possibility exists that betacellulin is primarily a ligand for another known member of the EGF receptor family, which includes the *erb*B2 (*neu/HER2*) (*25*) and *erb*B3 oncogene products (*26*). However, our analyses of ligand-dependent tyrosine phosphorylation indicate that the *erb*B2 receptor is not stimulated by betacellulin (*22*). Thus, *erb*B3 or another receptor remain as candidates for the primary betacellulin receptor. Finally, the fact that betacellulin is mitogenic for smooth muscle and retinal pigment epithelial cells and is produced by proliferating pancreatic β cells suggests the possibility that release of betacellulin from β cells could have a role in the vascular complications associated with diabetes.

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SH2-Containing Phosphotyrosine Phosphatase as a Target of Protein-Tyrosine Kinases

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A mouse phosphotyrosine phosphatase containing two Src homology 2 (SH2) domains, Syp, was identified. Syp bound to autophosphorylated epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) receptors through its SH2 domains and was rapidly phosphorylated on tyrosine in PDGF- and EGF-stimulated cells. Furthermore, Syp was constitutively phosphorylated on tyrosine in cells transformed by v-*src*. This mammalian phosphatase is most closely related, especially in its SH2 domains, to the *corkscrew* (*csw*) gene product of *Drosophila*, which is required for signal transduction downstream of the Torso receptor tyrosine kinase. The *Syp* gene is widely expressed throughout embryonic mouse development and in adult tissues. Thus, Syp may function in mammalian embryonic development and as a common target of both receptor and nonreceptor tyrosine kinases.

Protein tyrosine phosphorylation is a principal biochemical mechanism by which hormones and growth factors regulate cell proliferation, differentiation, and metabolism. Activated receptor protein-tyrosine kinases (PTKs) apparently stimulate intracellular signaling pathways by binding and phosphorylating regulatory cytoplasmic proteins (*1–5*). These cytoplasmic targets share a common structural element, SH2 domain, that mediates their association with specific phosphotyrosine-containing sites on autophosphorylated receptors and thereby controls the initial interactions of receptors with their substrates (*6*). Because cell growth and development are tightly controlled physiological processes, dephos-

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phorylation of phosphotyrosine residues by phosphotyrosine phosphatases (PTP) is also likely to be highly regulated (*7*). A mammalian PTP containing two SH2 domains has been identified (variously named PTP1C, SH-PTP1, HCP, and SHP), raising the possibility of a direct interplay between PTKs and PTPs (*8*). However, expression of this PTP is largely confined to hematopoietic cells (*8*), and its role in the regulation of cell signaling processes is unknown. Accordingly, we have pursued the isolation of more widely expressed SH2-containing PTPs.

We designed a cloning strategy that made use of the polymerase chain reaction (PCR) (*9*). We amplified cDNA derived from the RNA of mouse A31 cells with two degenerate oligonucleotide primers, one based on a conserved SH2 sequence FLVRES (*6*) and the other on a conserved PTP motif DFWXMXW (F, Phe; L, Leu; V, Val; R, Arg; E, Glu; S, Ser; D, Asp; W, Trp; X, any amino acid; M, Met) (*10*). A PCR product of 0.5 kb was generated by this procedure which, when cloned and sequenced, proved to encode a previously undescribed SH2 domain. This sequence was then used as a probe to screen a cDNA library from the mouse mammary carcinoma cell line FM3A (*11*). Four overlapping cDNA clones encompassing 2031 nucleotides were isolated, which in composite gave a single open reading frame encoding a polypeptide of 585 amino acids (Fig. 1A). The presumptive ATG translational initiation site is preceded by several in-frame stop codons. Consistent

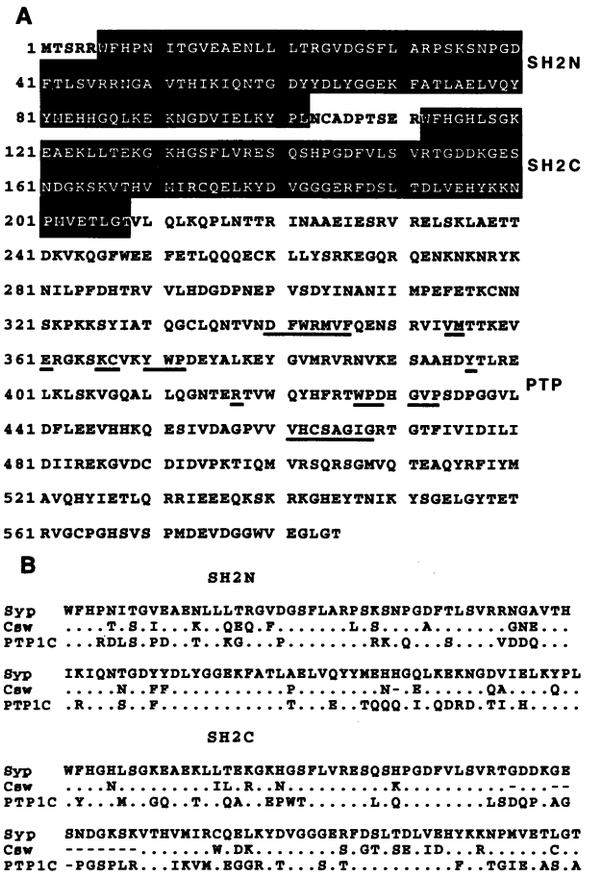

Fig. 1. Amino acid sequence of Syp and comparison with Csw and PTP1C. (**A**) The deduced 585–amino acid sequence of Syp. The two SH2 domains (SH2N and SH2C) are boxed. Conserved residues in the COOH-terminal PTP catalytic domain are underlined. The GenBank accession number for the *Syp* nucleotide sequence is L08663. (**B**) Comparison of the NH_2- and COOH-terminal SH2 domains (SH2N and SH2C) of Syp, Csw, and PTP1C. Dots represent amino acids in Csw and PTP1C that are identical to those found in Syp. Dashes in Csw and PTP1C represent gaps introduced for optimal alignment. (**C**) Comparison of the PTP catalytic domains of Syp, Csw, and PTP1C. The PTPase insert in Csw is not shown (*18*).

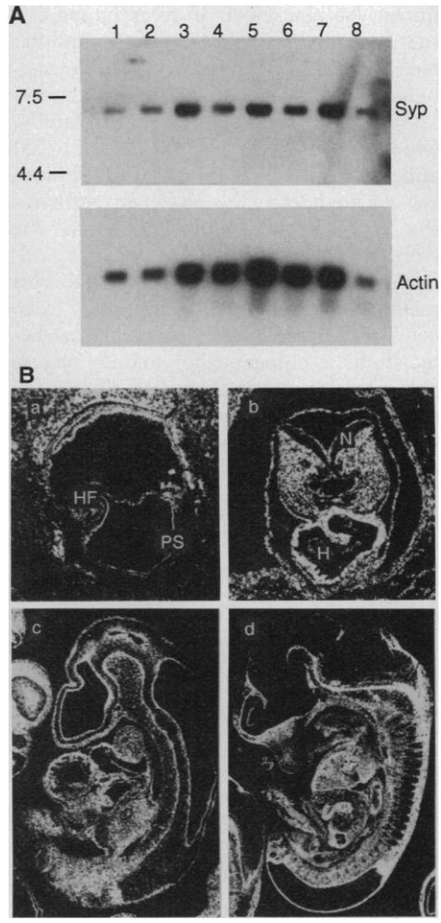

Fig. 2. Expression of *Syp* RNA in mouse embryos. (**A**) Polyadenylated mRNA was isolated from mouse embryos at days 9.5 to 16.5 and subjected to Northern blot analysis with the 2-kb composite *Syp* cDNA (*11*) as a probe. A mouse β-actin probe was also used as a control for the amount of RNA and its integrity. Lanes 1 through 8: 9.5, 10.5, 11.5, 12.5, 13.5, 14.5, 15.5, and 16.5 days of embryogenesis, respectively. Molecular sizes are indicated at the left (in kilobases). (**B**) ^{35}S-labeled sense and antisense RNA probes were synthesized by in vitro transcription of the 2.0-kb composite *Syp* cDNA (*11*) cloned in pBluescript and used for in situ hybridization as described (*20*). Panels (a) through (d), dark-field illuminations of sections hybridized with the antisense *Syp* probe of (a) a sagittal section through a head-fold stage embryo (d7.5); (b) transverse section through an early somite stage embryo (d8.5); (c) sagittal section of a d9.5 embryo; and (d) sagittal section of a d12.5 embryo. In parallel experiments, the *Syp* sense probe gave no specific signals. The apparently weaker hybridization signal in neural tissue is due to quenching by the counterstain. Silver grains were clearly visible under bright-field optics. HF, head fold; PS, primitive streak; N, neural fold; and H, heart.

with this prediction, in vitro transcription and translation of the composite *Syp* cDNA produced a polypeptide of 65 kD that was specifically recognized by antibodies to bacterial glutathione S-transferase (GST) fusion proteins containing either the Syp SH2 domains or the Syp PTP domain (*12*).

At its NH_2-terminus, Syp contains two SH2 domains with residues that are highly conserved among other SH2 domains and participate in recognition of phosphotyrosine (*6*, *13*). An exception is a conserved arginine located in the α1 helix of the Src SH2 domain, which is replaced by a glycine in both Syp SH2 domains (Gly^{13} and Gly^{119}) and in the SH2 domains of PTP1C (*8*). Although the arginine normally found at this position is involved in the recognition of phosphotyrosine through interaction of its amino group with the tyrosine ring, it is apparently not essential for SH2 binding to proteins containing phosphotyrosine (*14*). Syp also contains all the amino acid residues diagnostic of the catalytic domains of PTPs (Fig. 1A). The overall structure of Syp is very similar to that of PTP1C and to the product of the *Drosophila* gene *corkscrew* (*csw*), which also encodes an SH2-containing PTP (*15*). Sequence comparisons revealed that mouse Syp is most closely related to *Drosophila* Csw, especially within its SH2 domains, excluding a 155–amino acid insert sequence in the Csw PTP domain that is missing from Syp (Table 1). Syp and Csw are more similar to one another than either is to mouse PTP1C.

Table 1. Similarity of the SH2 and PTPase domains of Syp, Csw, and PTP1C. I, percentage of amino acid residues that are identical; S, percentage of amino acids that are similar. The following were considered conservative changes: R/K; E/D; T/S; V/I/L/A; and Y/F (*19*).

Comparison	SH2N		SH2C		PTPase	
	I	S	I	S	I	S
Syp-Csw	75	80	71	80	55	65
Syp-PTP1C	64	75	53	61	57	67
Csw-PTP1C	60	66	44	60	49	59

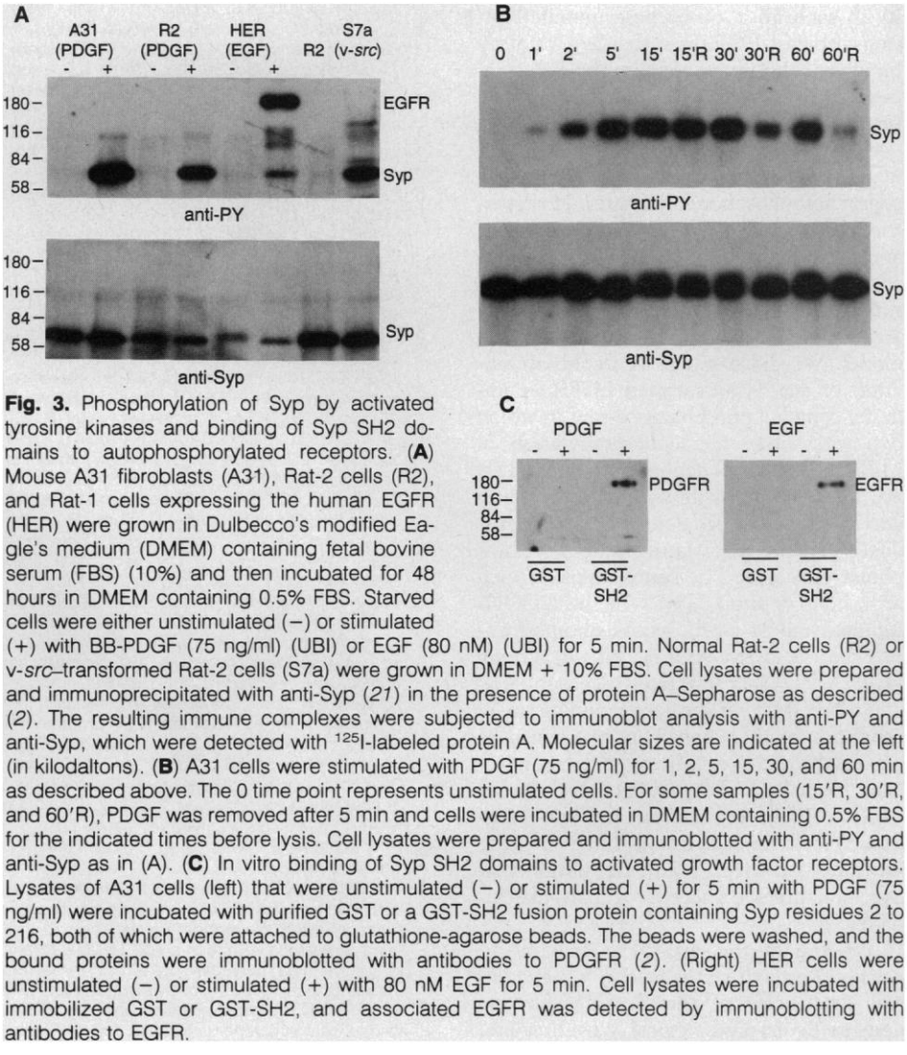

Fig. 3. Phosphorylation of Syp by activated tyrosine kinases and binding of Syp SH2 domains to autophosphorylated receptors. (**A**) Mouse A31 fibroblasts (A31), Rat-2 cells (R2), and Rat-1 cells expressing the human EGFR (HER) were grown in Dulbecco's modified Eagle's medium (DMEM) containing fetal bovine serum (FBS) (10%) and then incubated for 48 hours in DMEM containing 0.5% FBS. Starved cells were either unstimulated (−) or stimulated (+) with BB-PDGF (75 ng/ml) (UBI) or EGF (80 nM) (UBI) for 5 min. Normal Rat-2 cells (R2) or v-*src*–transformed Rat-2 cells (S7a) were grown in DMEM + 10% FBS. Cell lysates were prepared and immunoprecipitated with anti-Syp (*21*) in the presence of protein A–Sepharose as described (*2*). The resulting immune complexes were subjected to immunoblot analysis with anti-PY and anti-Syp, which were detected with ^{125}I-labeled protein A. Molecular sizes are indicated at the left (in kilodaltons). (**B**) A31 cells were stimulated with PDGF (75 ng/ml) for 1, 2, 5, 15, 30, and 60 min as described above. The 0 time point represents unstimulated cells. For some samples (15′R, 30′R, and 60′R), PDGF was removed after 5 min and cells were incubated in DMEM containing 0.5% FBS for the indicated times before lysis. Cell lysates were prepared and immunoblotted with anti-PY and anti-Syp as in (A). (**C**) In vitro binding of Syp SH2 domains to activated growth factor receptors. Lysates of A31 cells (left) that were unstimulated (−) or stimulated (+) for 5 min with PDGF (75 ng/ml) were incubated with purified GST or a GST-SH2 fusion protein containing Syp residues 2 to 216, both of which were attached to glutathione-agarose beads. The beads were washed, and the bound proteins were immunoblotted with antibodies to PDGFR (*2*). (Right) HER cells were unstimulated (−) or stimulated (+) with 80 nM EGF for 5 min. Cell lysates were incubated with immobilized GST or GST-SH2, and associated EGFR was detected by immunoblotting with antibodies to EGFR.

Northern blot analysis of embryonic mouse RNA identified a single 7.0-kb *Syp* transcript that was highly expressed in mouse embryos at the stages analyzed, days 9.5 to 18.5 [Fig. 2A and (*12*)]. To examine *Syp* expression during mouse embryogenesis in more detail, we used in situ RNA hybridization analysis of early mouse embryos (days 7.5 to 15.5). At day 7.5, *Syp* RNA was detected in both embryonic and extraembryonic tissues and in the maternal tissues (Fig. 2B). Expression continued to be widespread at all later stages examined, with small variations in the amount of expression between tissues. For example, a relatively large amount of *Syp* RNA was present in the heart, whereas smaller amounts were detected in the neural ectoderm and nervous system. *Syp* RNA was also detected in various adult tissues, including brain, heart, kidney, liver, lung, and spleen. The highest amounts were present in brain and heart (*12*). The abundant and broad expression of *Syp* in the early mouse embryo suggests that it may be necessary for normal development. Mutant analysis has demonstrated that *csw* is specifically required for the formation of terminal structures of the *Drosophila* embryo, although it is expressed throughout the embryo (*15*).

Csw functions downstream of the Torso receptor tyrosine kinase and transduces a signal from Torso to effectors that participate in terminal formation in the *Drosophila* embryo (*15*). However, there is no direct biochemical evidence to show that Csw is directly regulated by or physically interacts with the Torso kinase. To test if Syp might be a downstream target of receptor tyrosine kinases in mouse cells, we treated A31 fibroblasts with PDGF for varying periods of time. Proteins from cell lysates were immunoprecipitated with antibodies to Syp (anti-Syp), and the resulting immune complexes were then blotted with anti-Syp or antibodies to phosphotyrosine (anti-PY) (Fig. 3, A and B). Tyrosine phosphorylation of the 65-kD Syp polypeptide was evident within 1 min after stimulation of cells with PDGF, reached a maximum within 5 to 15 min, and remained high for at least 60 min in the presence of PDGF (Fig. 3B). If PDGF was removed from A31 cells after 5 min of treatment, Syp was slowly dephosphorylated. PDGF stimulation of Rat-2 cells also induced phosphorylation of Syp on tyrosine (Fig. 3A). Syp phosphorylation was examined directly by metabolic labeling of A31 cells with ^{32}P. Syp was poorly phosphorylated in unstimulated cells; however, PDGF stimulation induced a marked increase in Syp phosphorylation. Phosphoamino acid analysis showed that Syp from PDGF-stim-

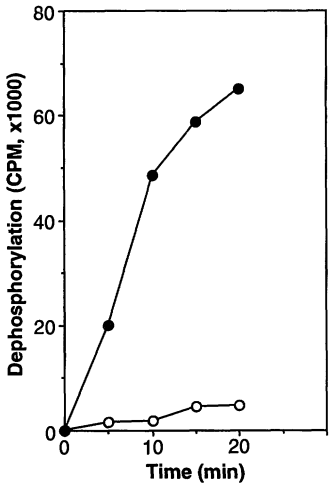

Fig. 4. Syp in vitro phosphatase activity. Equal amounts of A31 cell lysates were immunoprecipitated with preimmune serum (○) or with anti-Syp (●). The immune complexes were then assayed for phosphatase activity. Syp was immunoprecipitated with the use of protein A–Sepharose beads as described for Fig. 3 except that the precipitates were washed three times with buffer containing 50 mM Hepes (pH 7.5), 150 mM NaCl, 1.5 mM $MgCl_2$, 10% glycerol, 1% Triton X-100, 1 mM EGTA, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, leupeptin (10 μg), and aprotinin (10 μg). The tyrosine phosphatase assay was performed as described (*16*). Bovine MBP (Sigma) was phosphorylated on tyrosine by the $p43^{v\text{-}abl}$ kinase (Oncogene Science) (*16*). Dephosphorylation of MBP was done at room temperature in buffer containing 50 mM imidazole (pH 7.5), 10 mM dithiothreitol, 5 mM EDTA, immunoprecipitated Syp, and 110,000 cpm of substrate in a final volume of 30 μl. The reaction was terminated by the addition of 200 μl of 20% ice-cold trichloroacetic acid. After centrifugation, ^{32}P in the supernatant was counted to measure the extent of dephosphorylation.

ulated cells was principally phosphorylated on tyrosine and contained lower amounts of phosphothreonine and phosphoserine (*12*).

To investigate whether Syp is a more general substrate for tyrosine kinases, we stimulated Rat-1 cells (HER) expressing the human epidermal growth factor receptor (EGFR) with EGF; incubation with EGF for 5 min induced tyrosine phosphorylation of Syp. Syp from EGF-stimulated HER cells coprecipitated with a 175-kD protein containing phosphotyrosine (Fig. 3A) that corresponds to the autophosphorylated EGF receptor as confirmed by immunoblotting with antibodies to the EGFR (*12*). Consistent with this finding, bacterially expressed Syp SH2 domains, synthesized as GST fusion proteins, bound to the activated platelet-derived growth factor receptor (PDGFR) and EGFR in vitro (Fig. 3C). These results suggest that autophosphorylated growth factor receptors can bind to Syp SH2 domains in vivo and subsequently phosphorylate Syp on tyrosine. These are the hallmarks of an SH2-mediated receptor-target interaction, suggesting that Syp may function downstream of multiple PTKs.

Transforming nonreceptor tyrosine kinases, such as v-Src, deliver a mitogenic signal that results in malignant cell growth. Although Syp was not phosphorylated on tyrosine in parental Rat-2 fibroblasts, it was highly and constitutively tyrosine phosphorylated in a Rat-2 cell line transformed with v-*src* (Fig. 3A). This suggests that Syp may also be a target of the v-Src oncoprotein. A number of other more minor phosphotyrosine-containing proteins coprecipitated with Syp from *v-src*–transformed S7a cells, as well as from EGF-stimulated cells.

We assayed Syp phosphatase activity in vitro using immunopurified Syp, with tyrosine-phosphorylated myelin basic protein (MBP) as a substrate (*16*). As shown in Fig. 4, Syp was associated with PTP activity in vitro. There was no significant difference between the in vitro phosphatase activities of endogenous Syp immunoprecipitated from unstimulated A31 cells or from PDGF-stimulated A31 cells (*12*). It is difficult, however, to assess the effect of tyrosine phosphorylation on Syp phosphatase activity in such an assay because immunoblotting with anti-PY showed that Syp was itself rapidly dephosphorylated in the reaction, possibly as a result of autodephosphorylation (*12*).

One possible role for Syp is to act as a negative regulator by dephosphorylating receptor autophosphorylation sites. However, the observation that Syp itself becomes, and remains, highly tyrosine phosphorylated in growth factor–stimulated cells and is stably associated with tyrosine-phosphorylated EGFR might argue against this simple model. We did not observe dephosphorylation of the Syp-associated EGFR in immune complex phosphatase assays in which Syp was active in dephosphorylation of MBP and was itself dephosphorylated (*12*). An alternative possibility is that Syp also acts as a positive regulator or signal transducer by dephosphorylating inhibitory phosphotyrosine sites. The best example of such an inhibitory site is Tyr^{527} in the COOH-terminal tail of c-Src, whose phosphorylation blocks c-Src tyrosine kinase activity (*17*). A PTP that selectively dephosphorylates c-Src Tyr^{527} or the corresponding site of other Src family members in vivo would be expected to have a positive effect on mitogenic signaling. The finding that Syp is phosphorylated on tyrosine in v-*src*–transformed cells and hence is likely a v-Src substrate is consistent with the notion that Syp can interact directly with Src-like kinases.

Binding of the Syp SH2 domains to activated growth factor receptors may control the access of the Syp phosphatase domain to its physiological substrates and stimulate Syp tyrosine phosphorylation. Tyrosine phosphorylation of Syp may induce its association with the SH2 domains of other tyrosine-phosphorylated proteins in vivo, which could then be rapidly dephosphorylated by the Syp catalytic domain. Several phosphotyrosine-containing proteins did coprecipitate with Syp from v-*src*–transformed and EGF-stimulated cells (Fig. 3A). Occupancy of the Syp phosphorylation sites by an SH2 domain might also block autodephosphorylation. These notions are amenable to biochemical and genetic investigation.

Note added in proof: The predicted product of the human *SH-PTP2* cDNA (*18*) is very similar to mouse Syp.

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9. For PCR, two degenerate oligonucleotides were used. The 5′ sense primer CACAAGCTT(T/C)XT-X(G/A)TXCGXGAXAG was based on the amino acid sequence FLVRES, which is conserved in SH2 domains (*6*). The 3′ antisense primer TCTG-GATCCAXXXCATXXXCCA(A/G)AA(A/G)TC was based on the sequence DFWXMXW, which is conserved in PTP domains [M. Streuli, N. X. Krueger, A. Y. M. Tsai, H. Saito, *Proc. Natl. Acad. Sci. U.S.A.* **86**, 8698 (1989); K. Guan, R. J. Deschenes, H. Qiu, J. E. Dixon, *J. Biol. Chem.* **266**, 12964 (1991); T. Yi, J. L. Cleveland, J. N. Ihle, *Blood* **78**, 2222 (1991)]. The underlined sequences are Hind III and Bam HI recognition sites, and X represents G, A, T, and C. Total RNA isolated from mouse Balb/c 3T3 cells (clone A31) was reverse-transcribed with the 3′ antisense primer and converted into double-stranded cDNA. The resulting cDNA was amplified with the two primers and the touchdown PCR program [R. H. Don *et al.*, *Nucleic Acids Res.* **19**, 4008 (1991)]. The PCR product was digested with Hind III and Bam HI, cloned into pBluescript KS vector (Stratagene), and sequenced by the dideoxy method with a DNA sequencing kit (Pharmacia) (*19*).
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11. The PCR product isolated as described above was labeled with ^{32}P-labeled deoxycytidine 5′-triphosphate by the random-priming method and used as a probe to screen a λgt11 cDNA library of mouse mammary carcinoma cell FM3A by standard techniques. Four positive clones (C7, 8, 12, and 14) were purified, and the cDNA inserts were subcloned into pBluescript KS vec-

tor and sequenced. The four cDNA inserts (C7, 8, 12, and 14) are 164, 1395, 1014, and 899 bp long, respectively. Two clones (C8 and C12) were then used to make a composite cDNA (2031 bp) and cloned into pBluescript KS vector. This composite cDNA insert covering the entire coding region of *Syp* was sequenced again on both DNA strands.
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19. Abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
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21. Antiserum to Syp was made as follows: A DNA sequence encoding the two SH2 domains of Syp (residues 2 to 216) was cloned into the pGEX kt expression vector, and the resulting GST fusion protein was isolated as described (*2*). New Zealand white rabbits were immunized with the purified GST-SH2 fusion proteins.
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Activation of a Phosphotyrosine Phosphatase by Tyrosine Phosphorylation

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Regulation of cell proliferation, differentiation, and metabolic homeostasis is associated with the phosphorylation and dephosphorylation of specific tyrosine residues of key regulatory proteins. The phosphotyrosine phosphatase 1D (PTP 1D) contains two amino terminally located Src homology 2 (SH2) domains and is similar to the *Drosophila corkscrew* gene product, which positively regulates the *torso* tyrosine kinase signal transduction pathway. PTP activity was found to be regulated by physical interaction with a protein tyrosine kinase. PTP 1D did not dephosphorylate receptor tyrosine kinases, despite the fact that it associated with the epidermal growth factor receptor and chimeric receptors containing the extracellular domain of the epidermal growth factor receptor and the cytoplasmic domain of either the HER2-*neu*, *kit*-SCF, or platelet-derived growth factor β (βPDGF) receptors. PTP 1D was phosphorylated on tyrosine in cells overexpressing the βPDGF receptor kinase and this tyrosine phosphorylation correlated with an enhancement of its catalytic activity. Thus, protein tyrosine kinases and phosphatases do not simply oppose each other's action; rather, they may work in concert to maintain a fine balance of effector activation needed for the regulation of cell growth and differentiation.

Protein phosphorylation and dephosphorylation are key events in the pathways that regulate cell growth and differentiation. These processes are triggered by secreted polypeptide growth factors or hormones that activate tyrosine kinases to induce specific cellular responses (*1*). The cellular factors that participate in these signaling pathways include polypeptide substrates that contain the Src-homology regions, SH2 and SH3, either alone or in combination with regions that provide enzymatic activity (*2*). The SH2 domain elements of such substrates bind with high affinity to specific phosphorylated tyrosine residues of activated tyrosine kinases and thereby initiate the subsequent intracellular signal transduction cascade (*1*). For example, phospholipase Cγ interacts through its SH2 domain with the cytoplasmic domain of a receptor tyrosine kinase (RTK) and is activated by subsequent phosphorylation (*3*). Protein tyrosine phosphatases (PTPs) are potential regulators of protein tyrosine kinases (PTKs), but it is not clear how these crucial components of phosphotyrosine signaling cascades are activated (*4*).

The large number of currently known members of the PTP family suggests that there may be specificity in PTP-RTK interactions. Recently, a cDNA encoding a PTP designated PTP 1C was cloned from several sources (*5*, *6*). In addition to a single catalytic domain, the structure of this PTP includes a pair of SH2 regions near the NH_2-terminus, which suggested that PTK activity might be regulated by SH2 domain–mediated interaction of tyrosine-phosphorylated RTKs with a PTP.

We identified a PTP, designated PTP 1D, that contains an SH2 domain and has extensive sequence similarity to PTP 1C (*7*). Overlapping clones from a cDNA library from SK-BR-3 mammary carcinoma cells covered 6.8 kb and included in the 5′ portion an open reading frame of 1779 bp that encoded a protein of 593 amino acids with a calculated molecular size of 68 kD (Fig. 1). The coding sequence was flanked by 129 nucleotides of 5′-untranslated sequence and approximately 4.9 kb of 3′-untranslated sequence. The predicted amino acid sequence contains a PTP catalytic domain at the COOH-terminus and two adjacent SH2 domains that are located in the NH_2-terminal portion. PTP 1D and PTP 1C are very similar in their overall structure, with only minor divergence toward the COOH-terminus. The two proteins exhibit 55% sequence identity and 71% overall similarity.

The recently characterized product of the *Drosophila corkscrew* (*csw*) locus (*8*), a gene that participates in signaling by the tyrosine kinase encoded by the *torso* gene (*9*), is similar to PTP 1C and PTP 1D in the SH2 and PTP domains. Within the sequences that could be aligned, *csw* was 69 and 76% similar to PTP 1C and PTP 1D,

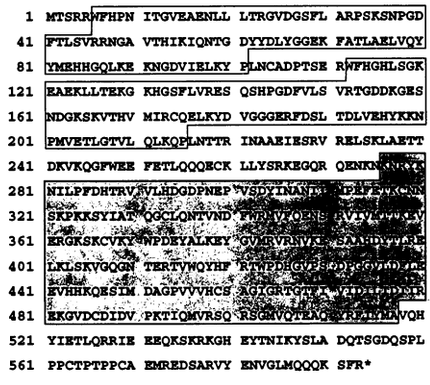

Fig. 1. Amino acid sequence of PTP 1D. The deduced amino acid sequence is shown. SH2 and PTP domains are boxed and the latter is shaded. The nucleotide sequence was determined with the chain termination method (*19*). Abbreviations for the amino acid residues are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

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