Src kinase associates with a member of a distinct subfamily of protein-tyrosine phosphatases containing an ezrin-like domain

(focal adhesion/cytoskeleton/src homology 2 and 3 domains)

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ABSTRACT A 6.2-kb full-length clone encoding a distinct protein-tyrosine phosphatase (PTP; EC 3.1.3.48), PTPD1, was isolated from a human skeletal muscle cDNA library. The cDNA encodes a protein of 1174 amino acids with N-terminal sequence homology to the ezrin-band 4.1–merlin–radixin protein family, which also includes the two PTPs H1 and MEG1. The PTP domain is positioned in the extreme C-terminal part of PTPD1, and there is an intervening sequence of about 580 residues without any apparent homology to known proteins separating the ezrin-like and the PTP domains. Thus, PTPD1 and the closely related, partially characterized, PTPD2 belong to the same family as PTPH1 and PTPMEG1, but because of distinct features constitute a different PTP subfamily. Northern blot analyses indicate that PTPD1 and PTPD2 are expressed in a variety of tissues. In transient coexpression experiments PTPD1 was found to be efficiently phosphorylated by and associated with the src kinase pp60^{src}.

Transient tyrosine phosphorylation of specific proteins is observed when mammalian cells communicate with their surroundings either through cytokines/hormones and their cognate receptors or by cell–cell/cell–substrate interactions. The latter type of cell communication *in vivo* is thought to be reflected, at least in part, by focal adhesion plaques, an *in vitro* phenomenon in which specific contact points are formed when fibroblasts grow on appropriate substrates (1–3). Several focal adhesion proteins are phosphorylated on tyrosine residues when fibroblasts adhere to and spread on extracellular matrix (4). However, aberrant tyrosine phosphorylation of these proteins can lead to cellular transformation. Thus, overexpression of the oncogene *v-src* leads to tyrosine phosphorylation of specific focal adhesion proteins, thereby disrupting focal adhesion with a permanently transformed phenotype as a result (2). The exact mechanism of this has not yet been defined, although several proteins have been identified as substrates for the pp60^{v-src} kinase.

The above-mentioned as well as many other studies indicate that there is a strong need for regulation of tyrosine kinases (TKs) through the counteracting activities of protein-tyrosine phosphatases (PTPs; EC 3.1.3.48). Indeed, the oncogenic action of TKs may be suppressed by PTPs. T-cell PTP, and especially a truncated form of this enzyme (5), can inhibit the transforming activity of *v-erbB* and *v-fms* (6, 7). Moreover, Brown-Shimer et al. (8) have shown that transformation by an oncogenic form of the *HER2/neu* gene was suppressed in NIH 3T3 fibroblasts overexpressing PTP1B. In this context, it seems significant that increased expression of PTP1B and LAR, but not T-cell PTP, was observed in mammary carcinoma cells overexpressing *HER2/neu* (9).

Recently, a more complex picture has emerged with respect to the function of PTPs in cellular differentiation and transformation. An alternative role for PTPs as promoters of cellular transformation has been illustrated by overexpression of the receptor PTP α in rat fibroblasts (10), leading to permanently increased activity of pp60^{src} and concomitant cell transformation and tumorigenesis. In accordance with these results, PTP α has been reported to enhance the kinase activity of pp60^{src} and to be involved in neuronal differentiation (11). Furthermore, overexpression of PTP mutants can cause dramatic changes in cell growth and differentiation. Cool et al. (5) have shown that expression of a truncated form of T-cell PTP results in a different and broader subcellular localization concomitant with a reduced growth rate and gross morphological changes including multinucleation of the cells. Likewise, overexpression of PTP1B leads to a substantial increase in the proportion of multinucleated cells (12).

An intimate association between PTPs and focal adhesion is supported by the finding that two related PTPs, PTPH1 (13) and PTPMEG1 (14), both contain an N-terminal domain with similarity to several proteins that are believed to act as links between the cell membrane and the cytoskeleton, including ezrin (15) and talin (16). Although the exact function of these PTPs has yet to be defined, it is likely that they participate in the fine tuning of tyrosine phosphorylation of focal adhesion and cytoskeletal proteins. Interestingly, it has been found that the PTP activity in the membranes is elevated considerably in density-dependent growth-arrested fibroblasts implicating PTPs in the regulation of contact inhibition (17). The substrates for this as-yet-unidentified PTP(s) have not been characterized, but it might be speculated that they belong to the class of focal adhesion proteins.

As part of an effort to define the specificity of action and the mechanisms of regulation of PTPs (6, 18), we here report the cDNA cloning and characterization of a PTP, PTPD1, which defines a subfamily of the ezrin-like domain-containing family of proteins. Interestingly, PTPD1 is a substrate of the src kinase and there is an association of the two molecules.

MATERIALS AND METHODS

Preparation of RNA. Total RNA was isolated from human tissues (obtained from the National Disease Research Interchange, Philadelphia) and cell lines by the guanidinium thiocyanate/CsCl procedure (19). Poly(A)⁺ RNA was isolated on an oligo(dT)–cellulose column.

Polymerase Chain Reaction. First-strand cDNA was synthesized from 2 μ g of poly(A)⁺ RNA by using oligo(dT) priming and Moloney murine leukemia virus RNase H-negative re-

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Abbreviations: PTP, protein-tyrosine phosphatase; SH2 and SH3 domains, src homology 2 and 3 domains.

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verse transcriptase from GIBCO/BRL in accordance with the supplier's recommendations.

cDNAs corresponding to PTPs expressed in skeletal muscle were isolated by PCR. In short, human muscle first-strand cDNA from above (corresponding to about 50 ng) was amplified with a set of degenerate oligonucleotide primers corresponding to the consensus sequences FWXMW and HCSAG(S/I/V)G, using the GeneAmp kit (Perkin-Elmer/Cetus). The fragments of the expected size (based on the structure of already-described PTPs) were isolated, subcloned by using the TA cloning system (Invitrogen), and sequenced with Sequenase (United States Biochemical) by the enzymatic chain termination method (20).

cDNA Library Construction and Screening. Five micrograms of human skeletal muscle poly(A)⁺ RNA was used for preparation of a Lambda ZAP II cDNA library (Stratagene) according to the supplier's instructions. A total of 2×10^6 independent plaques were screened for PTPD1-positive clones by using standard filter hybridization techniques. Fourteen positive clones were identified, isolated, excised *in vivo*, and analyzed. The longest of these clones (6.2 kb) was sequenced by the enzymatic chain-termination method with synthetic oligonucleotide primers based on existing sequences (20).

Northern Blot Analysis. Two-microgram samples of poly(A)⁺ RNA from various human tissues and cell lines were loaded in each lane, fractionated on a 1.0% agarose gel containing 0.66 M formaldehyde, and transferred to nylon membranes (Flash nylon, Stratagene). The blots were hybridized with a ³²P-labeled PTPD1 cDNA *Stu* I/*Eco*RI fragment (corresponding to 394 bp of the C-terminal half of the PTP domain and about 1 kb of the 3' untranslated region) and exposed to x-ray film with intensifying screens at -80°C. The filters were reprobed with the ³²P-labeled PTPD2 PCR fragment.

Transient Expression, Western Blotting, and Immunoprecipitation. The fibroblast cell line 293 (ATCC CRL 1573) was used for transient expression experiments as described in detail previously (6, 21). For these experiments, transfections were performed with the following cDNAs introduced into a pCMV expression plasmid: PTPD1, *v-src*, and chicken *c-src*. The expression plasmids were transfected either individually or in combinations. Four micrograms of plasmid DNA was transfected into 3×10^5 cells in 10-cm² wells according to the procedure described by Chen and Okayama (22). Eighteen hours later the medium was changed to low serum, and after a further 10 hr the medium was changed to L-[³⁵S]methionine-containing medium overnight. The cells were lysed in 0.2 ml of lysis buffer and aliquots were used for analysis of phosphotyrosine content by immunoblotting with anti-phosphotyrosine antibody 5E.2 (23). The expression levels of PTPD1 and *c-src* were analyzed by immunoblotting using rabbit polyclonal anti-peptide antibodies directed either against the PTPD1 peptide sequence QEVSEPLTAARHAQ (residues 618–631) or the C terminus of the *src* protein (LEDYFTSTEPQYQGENL). With the residual lysate an immunoprecipitation was performed with rabbit polyclonal antiserum directed against the C terminus of the *src* protein as described (6, 21). The samples were subjected to SDS/PAGE and the dried gels were autoradiographed.

RESULTS

We have used the PCR approach to investigate the expression of PTPs in various tissues. From human skeletal muscle we isolated two cDNA fragments with a high degree of sequence identity, which were designated PTPD1 and PTPD2. The predicted amino acid sequences, which contain many of the hallmarks of classical PTPs (24), are shown in Fig. 1A. PTPD1 and PTPD2 were compared with the most related PTPs by using the GAP program (25), with PTPH1 (13), PTP1C (26), and CD45 domain 1 (27) exhibiting the highest

degree of identity (35–39%), and PTPMEG1 (14), LAR domain 2 (28), and PTP1B (29) a lower degree (28–33%). However, their striking homology to each other (76% identity) clearly indicates that they define a distinct subgroup of PTPs, which we denote the PTPD subfamily.

Characterization of PTPD1 cDNA. A full-length cDNA clone of PTPD1 (6.2 kb) was isolated from a human skeletal muscle cDNA library and characterized (Fig. 1B). Downstream of two in-frame stop codons, an ATG codon is found at nucleotides 333–335 which conforms with a consensus translation initiation sequence (31). The predicted PTPD1 protein, which contains neither a signal sequence nor a transmembrane domain, consists of 1174 amino acids with a calculated molecular mass of 133 kDa. There is only one PTP domain, which is located in the extreme C-terminal part of the molecule. The N-terminal part shows similarity to the ezrin-radixin-band 4.1 protein family (15, 32, 33), suggesting that PTPD1 belongs to the class of intracellular PTPs with an overall structure similar

FIG. 1. (A) Predicted amino acid sequences encoded by cDNA fragments PTPD1 and PTPD2, identified by the PCR. Only residues differing from PTPD1 are shown for PTPD2. The sequences corresponding to the PCR primers are not included. (B) Predicted amino acid sequence of full-length PTPD1. The N-terminal ezrin-like domain and the PTP domain (shaded) are boxed. The sequence of the PCR fragment is in brackets. The putative *src* homology 3 (SH3) domain binding motif (solid bar underneath) and the acidic stretch (hatched bar underneath) are indicated. Asterisks indicate PYX motifs. Putative *src* homology 2 (SH2) domain binding motifs are doubly underlined.

FIG. 2. Comparison of PTPD1, PTPH1, and PTPMEG1. (A) Schematic representation showing the ezrin-like domains (solid box), the intervening segments (open box), and the PTP domains (hatched box). Asterisks indicate the PYX motif; Pro, the putative SH3 binding motif; and Ac, the acidic stretch. (B) Alignment of the ezrin-like domains. (C) Alignment of the PTP domains. Both alignments were calculated by the CLUSTAL program. Identical and conserved residues are highlighted.

to that of PTPH1 (13) and PTPMEG1 (14), as shown schematically in Fig. 2A.

The N-terminal portion of PTPD1 (about 310 residues) shows similarity to homologous regions of proteins which are thought to act as links between the cytoskeleton and the cell membrane: band 4.1 (32), ezrin (15), moesin (34), and merlin (35). Interestingly, similar regions have been described in two PTPs, PTPH1 (13), and PTPMEG1 (14). A comparison of the predicted amino acid sequence of the N-terminal part of PTPD1 with the homologous regions of PTPH1 and PTPMEG1 is shown in Fig. 2B. Although many features have been conserved among the three PTPs, it is apparent that PTPH1 and PTPMEG1 are more closely related to each other than to PTPD1. The ezrin-like domain of PTPD1 shows about 30% identity to the homologous domains of PTPH1 and PTPMEG1, whereas PTPH1 and PTPMEG1 share 55% identity in this

region. In addition, two putative src SH2 binding sites may be assigned in the ezrin-like domain of PTPD1: Y¹⁵⁸ESQ and Y²¹⁷GEE (36).

Despite the close relationship between the two PTP subfamilies they differ significantly in the intervening sequence that separates the ezrin-like and PTP domains, which in PTPD1 is about 150 amino acids longer than in PTPH1 and PTPMEG1 (Fig. 2A). PTPH1 and PTPMEG1 are about 45% identical in this region, and a homology analysis with the ALIGN program (26) gives a score of 37.0 (with a gap penalty of 10 and 100 random runs; a score of more than 5 is indicative of homology) (13). The scores for PTPD1 are 0.0 and 0.36 when compared with PTPH1 and PTPMEG1. There are several consensus sites for casein II kinase phosphorylation and many tyrosine residues throughout the molecule which might serve as targets for tyrosine kinases. The sequence motif PYX,

FIG. 3. Expression of PTPD1 mRNA in various human tissues analyzed by Northern blot hybridization. Each lane of the blot contains 2 μ g of poly(A)⁺ RNA. Size markers (kb) are indicated.

where X is a nonpolar amino acid (A, L, V, or P), is found 5 times in the intervening region and once at the boundary to the ezrin-like domain. Further, there are two relatively short stretches which might promote the binding of specific molecules to PTPD1. One is PPPYPYPPRP (residues 565–574) which resembles the consensus sequence for SH3 domain binding proteins (37). The other is the very acidic region EEEEEDEFEFE (residues 712–722) which could bind to positively charged target sequences on other proteins. Additionally, the sequence PQPYVMPPPP (residues 334–343) at the boundary between the ezrin-like domain and the intervening part might have SH3 domain binding capacity.

As indicated above, PTPD1 contains most of the hallmarks of PTPs. The catalytically essential cysteine-1108 and its flanking residues, VHCSAGVGRTG, are completely conserved (Fig. 1B and Fig. 2C). Surprisingly, another cysteine residue conserved in the consensus sequence KCXXYWP in classical PTPs is replaced with a serine residue in both PTPD1 (position 1007) and PTPD2.

An alignment of the PTP domains of PTPD1, PTPH1, and PTPMEG1 is given in Fig. 2C. PTPD1 is 41% and 38% identical to PTPH1 and PTPMEG1 in the PTP domain, respectively. In contrast, PTPH1 and PTPMEG1 are found to be 64% identical in the PTP domain, directly indicating a very close relationship between these two PTPs. As shown in Fig. 2C, the enzyme domain of PTPD1 contains three insertions in comparison with PTPH1 and PTPMEG1, two of which are also found in the PTPD2 sequence. The most C-terminal of these shows no homology between PTPD1 and PTPD2, which raises the possibility that these insertions determine in part the interaction with substrates and thereby the functional specificity of the enzymes.

FIG. 4. Transient expression of src kinases and PTPD1. v-src and c-src were transiently overexpressed in 293 cells either alone or together with PTPD1. Transfected cells were labeled metabolically with L-[³⁵S]methionine and lysed, and the cleared lysates were used for further analysis. (A) An aliquot of the lysate was analyzed by SDS/PAGE and immunoblotting with anti-phosphotyrosine antibody. Size markers (kDa) are indicated. (B) Expression control: Reblotting of the same filter with polyclonal antibodies directed against PTPD1 and c-src. (C) Immunoprecipitation of the residual lysate was performed with a rabbit antibody directed against the C terminus of c-src, and the immunoprecipitates were analyzed by SDS/PAGE followed by autoradiography.

Tissue Distribution Analysis. The expression pattern of PTPD1 and PTPD2 in various human tissues and cell lines was analyzed by Northern blots (Fig. 3 and data not shown). PTPD1 mRNA is expressed abundantly in human placenta, lung, and normal and diabetic skeletal muscle, and to a lesser extent in kidney and colon. Very low levels can be detected in liver and stomach after longer exposure, whereas spleen does not express detectable amounts of PTPD1. A similar tissue distribution pattern is found for PTPD2 with a major transcript size of about 11 kb (data not shown). Expression levels of both PTPD1 and PTPD2 are relatively high in the rhabdomyosarcoma cell lines RD (ATCC CCL 136) and A673 (ATCC CRL 1598). The PTPD1 mRNA level is considerably higher in the embryonic cell line RD than in A673, whereas the opposite is the case for PTPD2.

PTPD1 Interaction with src. To approach functional aspects of PTPD1 we employed transient expression in human 293 embryonic fibroblasts using a cytomegalovirus-based mammalian expression vector (6, 21). PTPD1 was coexpressed with either v-src or c-src. Cell lysates were analyzed by SDS/PAGE and Western blotting. Fig. 4A shows that the src kinase when transfected alone phosphorylates a broad range of proteins. However, when src kinases are cotransfected with PTPD1 an additional band of 130 kDa corresponding to PTPD1 (Fig. 4B) is heavily tyrosine phosphorylated. Moreover, PTPD1 was tightly bound to pp60^{src} as demonstrated by coimmunoprecipitation with an antibody directed against the C terminus of the src kinase, which is not present in the v-src protein (Fig. 4C).

DISCUSSION

Using PCR, we have identified two PTPs which define a subfamily of this group of enzymes. A full-length clone corresponding to one of these, PTPD1, was isolated and characterized. PTPD1 consists of three domains. The C-terminal portion is a classical PTP domain with the hallmarks of a bona fide PTP. The N-terminal region is similar to a domain found in ezrin and several other proteins, including two recently identified PTPs, PTPH1 and PTPMEG1. The intervening sequence does not show strong homology to any known protein. Although we do not have formal proof at this point, we predict that PTPD2, because of its striking homology to PTPD1, has a similar overall structure.

In classical PTPs, the cysteine residue in the consensus sequence KCXXYWP is highly conserved. Surprisingly, this cysteine is replaced with a serine residue in PTPD1 and PTPD2. However, it is unlikely that this substitution has significant impact on the intrinsic enzyme activity, since this motif is not found in several nonclassical protein tyrosine phosphatases such as VH1 and cdc25 for which enzyme activity is well documented (38, 39). Also, mutational analysis of this residue in the N-terminal PTP domain of LAR indicates that its contribution to the intrinsic activity is rather small (40). On the other hand, since cysteine residues for structural reasons are normally well conserved among related proteins, it is likely that the substitution is of significance for the overall folding of PTPD1 and thereby for the interaction with and specificity for protein substrates. It is therefore anticipated that PTPD1 has a distinct substrate specificity. In fact, transiently expressed and immunoprecipitated PTPD1 does not hydrolyze *p*-nitrophenyl phosphate under standard conditions, whereas it has significant, although low, activity towards ³²P-labeled poly(Glu,Tyr) (data not shown).

Even though PTPD1, PTPH1, and PTPMEG1 all have the same overall structure, it is obvious from sequence alignment analyses that PTPH1 and PTPMEG1 belong to one subfamily, PTPD1 and PTPD2 to another. First, PTPH1 and PTPMEG1 are 64% identical in the PTP domain, whereas PTPD1 only shows 41% and 38% identity to PTPH1 and PTPMEG1 in this

region. The PCR fragments encompassing portions of the catalytic domains of PTPD1 and PTPD2 are 76% identical. Second, similar relationships are found in the N-terminal, ezrin-like, domain. Third, the intervening sequence of PTPD1 is about 150 amino acids longer and does not show significant structural similarity to the corresponding regions in PTPH1 and PTPMEG1, which are about 45% identical.

PTPD1 has several structural features which point to specific protein-protein interactions and a defined subcellular localization. As already pointed out for PTPH1 (13) and PTPMEG1 (14), the N-terminal ezrin-like domain is likely to localize PTPD1 to the cell membrane, since it belongs to a class of proteins which seem to anchor the cytoskeleton to the plasma membrane. Our coexpression studies, furthermore, show that there is a strong association between tyrosine-phosphorylated PTPD1 and pp60^{src}. This interaction could be mediated by binding of the SH2 domain to two putative binding motifs found in the ezrin-like domain of PTPD1. Likewise, the PYX motif, which appears several times in the intervening segment, may represent a specific phosphorylation site and could promote binding to SH2 domains. Alternatively, this association could also be mediated by the proline-rich region, which is similar to defined SH3 domain binding structures in other proteins (37). In this context it is of interest that several cytoskeletal proteins have been found to contain SH3 domains (41), and at least one, tensin, to contain an SH2 domain (42).

At least three different types of stimuli lead to the phosphorylation of cellular proteins in the molecular mass range of 110–130 kDa: (i) clustering of β_1 integrins by antibodies or cell adhesion; (ii) activation of the receptors for bombesin, vasopressin, and endothelin; and (iii) transformation by pp60^{v-src} (43–45). The focal adhesion kinase (pp125^{FAK}) has been found to be a major component of this complex of phosphorylated proteins (46–48). Further, in the case of cell adhesion and of cell transformation by pp60^{v-src} the changes in phosphorylation correlate with increased pp125^{FAK} tyrosine kinase activity (49). However, other proteins in this size range which are phosphorylated by the src kinase have not yet been characterized. Two of these phosphotyrosine-containing proteins, pp110 and pp130, were found to associate stably with activated pp60^{src} (50, 51).

In summary, the molecular weight of PTPD1, its primary structure with an ezrin-like domain and a putative SH3 domain binding sequence, and its phosphorylation by and interaction with the src kinase suggest that this PTP subfamily might be involved in the control of tyrosine phosphorylation events in focal adhesions. The cloning of PTPD1 will allow us to test this hypothesis and to address questions relating to the specific function of this class of PTPs.

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