Protein-tyrosine Phosphatase D1, a Potential Regulator and Effector for Tec Family Kinases*

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ETK, also known as Bmx, is a member of the Tec tyrosine kinase family, which is characterized by a multimodular structure including a pleckstrin homology (PH) domain, an SH3 domain, an SH2 domain, and a catalytic domain. The signaling mechanisms regulating Etk kinase activity remain largely unknown. To identify factor(s) regulating Etk activity, we used the PH domain and a linker region of Etk as a bait for a yeast two-hybrid screen. Three independent clones encoding protein-tyrosine phosphatase D1 (PTPD1) fragments were isolated. The binding of PTPD1 to Etk is specific since PTPD1 cannot associate with either the Akt PH domain or lamin. In vitro and in vivo binding studies demonstrated that PTPD1 can interact with Etk and that residues 726–848 of PTPD1 are essential for this interaction. Deletion analysis of Etk indicated that the PH domain is essential for PTPD1 interaction. Furthermore, the Etk-PTPD1 interaction stimulated the kinase activity of Etk, resulting in an increased phosphorylation content in both factors. The Etk-PTPD1 interaction also increased Stat3 activation. The effect of PTPD1 on Etk activation is specific since PTPD1 cannot potentiate Jak2 activity upon Stat3 activation. In addition, Tec (but not Btk) kinase can also be activated by PTPD1. Taken together, these findings indicate that PTPD1 can selectively associate with and stimulate Tec family kinases and modulate Stat3 activation.

Tyrosine kinases play important roles in a variety of signaling cascades in many cell types. Tec tyrosine kinases, a new class of non-receptor tyrosine kinase, are an emerging family of proteins that are expressed in both hematopoietic and non-hematopoietic tissues. This family consists of the Btk (1, 2), Ikt (3, 4), Tec (5), and Etk/Bmx (6, 7) tyrosine kinases with closely homologous structures that include an N-terminal pleckstrin homology (PH) domain, followed by Tec homology (TH), SH3, SH2, and tyrosine kinase domains. These Tec kinases have been implicated in the signaling pathways of a variety of hematopoietic and antigen receptors. For instance, Btk has been found to be activated through B-cell receptor stimulation, the interleukin-5 receptor, gp130, and the mast cell Fce receptor (8–12). Mutations in Btk are associated with the human disease X-linked agammaglobulinemia as well as murine X-linked immunodeficiency (1, 13). In both cases, B-cell signaling is defective, and B-cell development is blocked (14, 15). Likewise, Itk plays a central role in T-cell signaling. Itk knockout mice have reduced numbers of mature thymocytes and show alterations in T-cell antigen receptor signaling (16). Tec itself is mainly involved in regulating pathways regulating myeloid growth and differentiation. In these cells, Tec is tyrosine-phosphorylated following cell stimulation by a variety of hematopoietic growth factors, such as interleukin-3, stem-cell factor, and granulocyte colony-stimulating factor (17–22), as well as lymphocyte surface antigens, such as CD3, CD28, CD38, and CD72 (23). Etk, unlike other members of the Tec kinase family, which are mostly hematopoietic cell-specific, is preferentially expressed in epithelial and endothelial cells (7). Thus, the Tec kinases and their roles in biological functions remain largely unknown.

Recently, Etk has been shown to mediate interleukin-6 signaling in the prostate cancer cell line LNCaP for differentiation (7) and Go12/13 signaling in NIH-3T3 fibroblasts for activation of serum response factor (24). In Btk-deficient DT40 cells, Etk can replace the function of Btk in activating phospholipase Cγ2 in response to B-cell receptor engagement (25). In rat liver epithelial cells, Etk can mediate cellular transformation of v-Src by activating Stat3 activity (26). Besides Stat3 activation, Etk has also been shown to activate Stat1 and Stat5 (27, 28). Recently, we have established an Etk-inducible system to demonstrate that Etk induces cyclin D1 reporter activation through Stat5 (28).

Although progress has been made in identifying signaling pathways lying upstream or downstream from members of the Tec kinase family, little is known about the signaling mechanisms regulating their kinase activity. Recent studies of Itk revealed that regulation of kinase activity is through an internal folding of the molecule (29). The intramolecular interactions of the SH3 and adjacent proline-rich domains lock the kinase in a closed, inactive state. Signaling molecules that contain proline-rich domains and phosphotyrosine can disrupt the intramolecular interactions, leading to the unfolding of the protein-tyrosine phosphatase D1; GST, glutathione S-transferase; HA, hemagglutinin; SIE, c-sis-inducible element; PTP, protein-tyrosine phosphatase.
kinase domain and allowing the phosphorylation sites to be exposed to Src family kinases. An analogy for this intramolecular regulation is found in the Src family kinases, in which phosphorylation of a carboxyl-terminal tyrosine residue is thought to regulate kinase activity through an intramolecular interaction with the SH2 domain (30, 31). Thus, activation of the kinase presumably involves dissociation of intramolecular interactions by signaling molecules, removing the constraint on the catalytic domain and producing an open, active kinase. Many investigators have attempted to understand the signaling mechanism of Tec family kinases by identifying ligands for the subdomains of these kinases. For example, the α subunit of Gα and Gαq proteins can directly stimulate the kinase activity of Btk via binding to the TH-SH3 and PH-TH regions of Btk (32–34), respectively. Thus, interaction with signaling molecules is likely a common mechanism for activation of Tec family kinases.

PH domains are primarily involved in protein-lipid or protein-protein interactions and regulate enzyme function by controlling interacting partners or cellular localization (35, 36). Studies of Tec family kinases have implied that the binding of lipid or protein signaling molecules to the PH domain is essential for activation and biological activity. Mutations in the Btk PH domain cause X-linked agammaglobulinemia and X-linked immunodeficiency (13, 37–39). One such mutation (R28C) has been shown to reduce the in vitro isositol polyphosphate- and phosphoinositide-binding activities of the isolated Btk PH domain (40–42). In contrast, the E41K mutation in the Btk PH domain results in an increase in membrane association and activation of Btk (16). Moreover, a recent study has indicated that phosphatidylinositol 3,4,5-trisphosphate binding with the PH domain acts as an upstream activation signal for Btk and Tec kinases (43). On the subject of protein interactions of the PH domain, G protein β/γ subunits (44); protein kinase C isoforms (27, 45, 46); BAP-135/TFII-I (47, 48); G protein α12, α13, and αs subunits (33); and actin filament (49) have been reported. Some protein interactions, such as Gα12/13q, result in activation of Btk kinase activity. Others, such as protein kinase C, lead to down-regulation of Btk and Etk activities. These findings suggest that the PH domain of Tec family kinases is a target site for interaction and regulation by signaling molecules.

We are interested in identifying the cellular signaling molecules for Etk. Etk contains PH, SH3, SH2, and kinase domains similar to those of other Tec family members. However, human Etk has an atypical TH motif that consists of a Btk motif and a unique sequence with two 22-amino acid repeats next to the SH3 domain. Since the Btk motif is highly homologous among the Tec family members, we included the Btk motif as part of the PH domain and designated a linker region L representing the region between the Btk motif and SH3 domains for Etk. To identify signaling factor(s) regulating Etk activity, we used a yeast two-hybrid system to screen a human heart two-hybrid cDNA library purchased from CLONTECH, and the yeast strain LA0 was kindly provided by Dr. S. Hollenberg (Volum Institute). Yeast two-hybrid screenings were performed as described (50). Interaction between Etk-PH-L and proteins encoded by the cDNA library will activate HIS3 and lacZ reporter genes. HIS3 confers upon yeast the ability to grow on histidine-free medium, whereas lacZ produces β-galactosidase that can be detected colorimetrically by filter assays.

**In Vitro Binding and Co-immunoprecipitation Assays**—GST fusion proteins were purified as described (51). 35S-Labeled proteins were made with the TNT reticulocyte lysate system (Promega). 35S-Labeled proteins were incubated with 2 μg of each GST fusion protein in 0.2 ml of binding buffer (10 mM Hepes (pH 7.5), 50 mM NaCl, 0.1% Nonidet P-40, 0.5 mM dithiothreitol, and 0.5 mM EDTA) for 1–2 h, washed four times, and analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. A fraction of the reaction mixture was analyzed by adding gel for analysis of the GST fusion proteins in yeast mating with a specific binding domain in mammalian cells, various HA-PTPD1 and T7-Etk constructs were transfected into 293T cells (10 cm) by the lipofection method. Thirty-six hours after transfection, cell were solubilized in 1 ml of lysis buffer (20 mM octyl glucoside, 0.5% Triton X-100, 0.3 mM NaCl, 25 mM sodium phosphate (pH 7.4), 0.02% NaN3, 2 mM sodium vanadate, 20 mM sodium pyrophosphate, 200 mM sodium fluoride, and protease inhibitor mixture (Complete; Roche Molecular Biochemicals)). Cell lysates were mixed with antiserum against HA (BAbCO, Richmond, CA) or against T7 (Novagen), and the immunocomplexes were collected on protein A-Sepharose beads (Amersham Pharmacia Biotech). Immunoblot analyses of precipitated proteins were performed as described previously (7). Anti-Stat3 and anti-phospho-Stat3 antibodies were purchased from New England Biolabs, Inc. (Beverly, MA). Antibodies to phosphotyrosine (4G10), Jak2, and c-Src were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY).

**Cell Culture, Transfection, and Reporter Gene Assay**—Human 293T or COS-7 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. Cells were seeded into 10-cm plates the day before transfection. Transfections were performed using LipofectAMINE (Life Technologies, Inc.) according to the manufacturer’s instruction. Cell extracts were harvested 36 h later for co-immunoprecipitation assays, kinase assays, and Western analysis. For the reporter gene assay, salivary Pa4 cells were transfected with the pLuCTKS3 reporter (a gift from Dr. Richard Jove) as well as the pH-Tk plasmid as an indicator for normalization of transfection efficiency. Luciferase activities (firefly luciferase for the reporter and Renilla luciferase for the indicator) were measured using the Dual-Luciferase™ assay system (Promega).

**In Vitro Kinase Assay**—The in vitro kinase assays of Etk were performed as described (34). Briefly, the immunoprecipitates were washed twice with kinase buffer (50 mM Tris (pH 7.0) and 2 mM MgCl2) and incubated with substrate buffer containing 50 mM Tris (pH 7.4), 2 mM MgCl2, 20 μM ATP, 5 μCi of 32P-Pi, and 2 μg of peptide substrate derived from the Btk SH3 sequence containing the major autophosphorylation site (34, 52). Following a 20-min incubation at 30 °C, the reactions were terminated by addition of 2× SDS sample buffer. The samples were boiled and loaded onto 20% SDS-polyacrylamide gels. The results were visualized and quantitated by phosphorimage analysis (Fuji Bio-imaging BAS1500 analyzer).

**Electrophoretic Mobility Shift Assay**—Electrophoretic mobility shift assay was performed according to the procedures described by Vignais et al. (53). Briefly, 6 μg of nuclear extracts were incubated with 50,000 cpm of 32P-labeled human SIE probe for 30 min at room temperature. The Stat3-SIE complex was resolved by electrophoresis on a 5% polyacrylamide gel in 0.5× Tris borate/EDTA. The specificity of the Stat3-SIE complex was further verified by adding anti-Stat3 antibody for supershift and unlabeled SIE for competition (data not shown).

**RESULTS**

**Protein-tyrosine Phosphatase D1 Interacts with Etk through the PH Domain**—To identify factors that can modulate Etk activity, we used a yeast two-hybrid system to screen a human heart two-hybrid cDNA library purchased from CLONTECH, and the yeast strain LA0 was kindly provided by Dr. S. Hollenberg (Volum Institute). Yeast two-hybrid screenings were performed as described (50). Interaction between Etk-PH-L and proteins encoded by the cDNA library will activate HIS3 and lacZ reporter genes. HIS3 confers upon yeast the ability to grow on histidine-free medium, whereas lacZ produces β-galactosidase that can be detected colorimetrically by filter assays.
heart cDNA library with a bait containing the PH domain and linker region fused to the LexA protein. From an initial screen of 2.3 x 10^7 primary yeast transformants, 264 positives were identified by simultaneous HIS3 and LacZ transcription. After subsequent purification and curing processes, plasmids of 45 strong interacting clones were isolated for sequence analysis. Three of these clones encode fragments of PTPD1 (as shown in Fig. 1A) fused in frame with the Gal4 transactivation domain. PTPD1 was initially identified as a non-receptor protein-tyrosine phosphatase (54). It encodes a protein of 1174 amino acids with an ezrin-like domain at the N terminus and a PTP domain at the C terminus separated by a long intervening sequence. All three isolated PTPD1 fragments are located in the intervening sequence (Fig. 1A). Two of these clones were further tested for binding specificity. As shown in Fig. 1B, both PTPD1 clones interacted specifically with the PH domain and linker region of Etk, as demonstrated by the formation of blue colonies. Notably, full-length Etk could also bind to both PTPD1 fragments, whereas one fragment containing residues 374–848 conferred a weak interaction as illustrated by a light-

Fig. 1. Interaction of PTPD1 and Etk in yeast and in vitro. A. Shown is a schematic drawing of Etk and PTPD1 and their derivatives used in the yeast two-hybrid assay. Full-length Etk, the PH domain and linker region (Etk-PH+L), and Etk with the PH domain and linker region deleted (EtkΔPH+L) were tagged at the NH2 terminus with LexA (shaded ovals). PTPD1 consists of an ezrin-like domain, an intervening segment, and the PTP domain. PK, protein kinase; Pro, putative SH3-binding motif; Ac, acidic stretch. The three PTPD1 fragments represent the clones isolated from the yeast two-hybrid screen of a human heart cDNA library linked to the Gal4 transactivation domain (GalAD) indicated by the black bars. The first and last amino acids of the fragments are numbered with respect to their positions in full-length PTPD1. B, PTPD1 specifically interacted with Etk in yeast two-hybrid tests. Yeast cells cotransformed with a bait (Etk segments or the control proteins lamin and Akt PH domain fused to the LexA protein) and a prey protein (PTPD1 fragments fused to the Gal4 activation domain or the Gal4 activation domain alone) were streaked on a 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal)-containing plate. The development of blue colonies indicates an interaction of proteins coded by the two plasmids. C, GST and in-frame GST fusion proteins with cDNA encoding the PH domain and linker region of Etk (GST-PH+L) were expressed in Escherichia coli and purified on glutathione-Sepharose bead columns. GST and fusion proteins were quantitated by Coomassie Blue staining of SDS-polyacrylamide gels, and equivalent protein amounts were used in the GST pull-down assay. In vitro transcription and translation of various 35S-labeled PTPD1 fragments were separately incubated with GST and GST-PH+L. After SDS-polyacrylamide gel electrophoresis, bound PTPD1 fragments were visualized by autoradiography (upper panel, lanes 4–9). Lanes 1–3 represent one-third of the amount of 35S-labeled PTPD1 fragments subjected to the GST pull-down assay as indicated by Input. Coomassie Blue-stained GST and GST-PH+L proteins from each sample were aligned to show protein levels (lower panel).
blue colony. In contrast, EtkΔ(PH+L) (lacking the PH domain and linker region) failed to produce the interaction. In addition, the interaction was specific since no appreciable interaction was detected with the PH domain of Akt or lamin as negative controls. These findings indicate that PTPD1 can specifically interact with Etk through the PH domain and linker.

To further confirm the interaction between Etk and PTPD1 in vitro, a GST pull-down assay was performed. An in vitro translated, 35S-labeled PTPD1 fragment containing residues 666-848 bound to the immobilized GST fusion protein with the PH domain and linker domain, but not to the immobilized GST protein (Fig. 2C, upper panel, lanes 4 and 7). Furthermore, full-length PTPD1 was also subjected to this assay, and it bound to the PH domain and linker region of Etk as well (data not shown). These findings are consistent with those of the yeast two-hybrid assay. PTPD1-(666-848) contains an acidic segment (residues 712-722) that might be involved in Etk interaction.

To further determine whether PTPD1 interacts with Etk in mammalian cells, human 293T cells were cotransfected with constructs expressing HA-PTPD1 and T7-Etk. Cell lysates were separately subjected to immunoprecipitation assays with anti-HA tag or anti-T7 tag antibody. HA-PTPD1 was detected in the immunocomplexes of Etk (Fig. 2A, lane 2). This interaction was also confirmed in a reciprocal co-immunoprecipitation assay as illustrated in Fig. 2B. Again, the Etk-PTPD1 interaction is dependent on residues 726-848 of PTPD1 since the deletion mutant HA-PTPD1Δ(726-848) cannot be detected in the immunocomplex of T7-Etk (Fig. 2A, lane 3). These observations indicate that Etk and PTPD1 can specifically form a complex in cells.

To further delineate the subdomain of Etk involved in PTPD1 binding, we used various deletion mutants of Etk, including the PH domain, linker region, SH3 domain, and SH2 domain, in co-immunoprecipitation experiments with PTPD1. The protein expression levels of each mutant and PTPD1 were very similar (Fig. 2C, middle panel). Notably, deletion of the PH domain resulted in loss of association with PTPD1, as demonstrated by lack of detection of the HA-PTPD1 protein in the anti-T7 tag immunoprecipitates (Fig. 2C, lane 4). Conversely, the linker region deletion mutant EtkΔL, like wild-type Etk, retained the interaction with PTPD1 (Fig. 2C, upper panel, lanes 2 and 6). Mutants EtkΔSH2 and EtkΔSH3 still associated with PTPD1, but to a lower extent. These observations were further verified by the reciprocal experiments with anti-HA immunoprecipitated complex and Western analysis with anti-T7 antibody (data not shown). These findings indicate that the PH domain of Etk is the major site for PTPD1 binding.

Etk-PTPD1 Interaction Increases the Tyrosine Phosphorylation of Both Factors—Since PTPD1 is a tyrosine phosphatase, it likely modulates the phosphorytrosine content of Etk. We next tested whether the Etk-PTPD1 interaction results in a change in the tyrosine phosphorylation of Etk. Interestingly, coexpression of wild-type PTPD1 increased the phosphorytrosine content of Etk (Fig. 2A, lower panel, lane 2). This consequence was due to the association with PTPD1 because the PTPD1 deletion mutant PTPD1Δ(726-848) failed to confer the effect (lane 3). Conversely, tyrosine phosphorylation of PTPD1 was also enhanced by interaction with Etk. This notion was also confirmed by the reciprocal experiment in which PTPD1 was immunoprecipitated by anti-HA antibody and Western blot analysis with 4G10 antibody (Fig. 2B, lower panel). To further establish the correlation between interaction and increased tyrosine phosphorylation of both Etk and PTPD1, Etk immunocomplexes from cell lysates expressing PTPD1 and various deletion mutants of Etk were subjected to Western analysis with 4G10 antibody. As illustrated in Fig. 2C (lower panel), only the EtkΔPH mutant failed to confer tyrosine phosphorylation of both factors. Taken together, these findings provide a direct correlation between Etk-PTPD1 association and increased tyrosine phosphorylation.

PTPD1 Stimulates the Kinase Activity of Etk—The increased tyrosine phosphorylation of both Etk and PTPD1 may result from the Etk kinase itself or other associated tyrosine kinases(s) in the Etk-PTPD1 immunoprecipitated complexes. To test
Fig. 3. The kinase activity of Etk is responsible for the tyrosine phosphorylation of PTPD1. 293T cells were transfected with pcDNA-HA-PTPD1 and pcDNA-T7-Etk (wild-type (WT)), pcDNA-T7-Etk K445Q, or pcDNA-T7-Etk Y566F. At 36 h post-transfection, the cells were lysed, and cell extracts were subjected to immunoprecipitation (IP) with anti-T7 antibody, followed by Western blotting (WB) with anti-HA antibody (upper panel), anti-phosphotyrosine antibody (anti-pY, middle panel), and anti-T7 antibody (lower panel). The expression level of PTPD1 in each fraction is also indicated.

Fig. 4. The phosphatase activity of PTPD1 is not involved in stimulating Etk kinase activity. 293T cells were transfected with pcDNA-T7-Etk and pcDNA-HA-PTPD1 (wild-type (WT)) or pcDNA-HA-PTPD1 C1108S. About 500 μg of the cell extracts were subjected to immunoprecipitation (IP) with anti-T7 antibody, followed by Western blotting (WB) with anti-HA antibody (upper panel), anti-phosphotyrosine antibody (anti-pY; middle panel), and anti-T7 antibody (lower panel).

These possibilities, a kinase-dead mutant of Etk, K445Q, in which lysine 445 in the ATP-binding pocket of Etk was mutated to glutamine, was subjected to co-immunoprecipitation experiments with PTPD1. In comparison with wild-type Etk, less PTPD1 was found in the complexes with Etk K445Q (Fig. 3, upper panel, lane 5). In addition, tyrosine phosphorylation of the immunocomplexes of Etk K445Q and PTPD1 was undetectable (middle panel). These findings indicate that the kinase activity of Etk is responsible for elevated tyrosine phosphorylation and that tyrosine phosphorylation of both factors increases their associations.

Autophosphorylation of a conserved tyrosine in the catalytic domain of Src kinase (Try416) and Btk kinase (Try551) is associated with an increase in their enzymatic activity (31, 55). Etk contains a highly conserved Btk-like phosphorylation site within its kinase domain (Try566). Mutation of this site will likely result in reduced Etk kinase activity. To further confirm that Etk kinase activity is critical to the elevated level of tyrosine phosphorylation, an Etk mutant (Etk Y566F) with tyrosine 566 converted to phenylalanine was assayed in a coexpression assay with PTPD1. Again, this mutant, similar to Etk K445Q, bound PTPD1 with less strength, and no tyrosine phosphorylation was found in the immunocomplexes of PTPD1 and Etk Y566F (Fig. 3, upper panel, lane 6). In contrast, other tyrosine residue mutants (Y294F and Y641F) that did not affect Etk autophosphorylation retained the ability to associate with PTPD1 and conferred increased tyrosine phosphorylation (data not shown). Thus, Etk intrinsic kinase activity is responsible for tyrosine phosphorylation of both factors and for the increased affinity between the factors.

Src family kinases can be positively regulated by several tyrosine phosphatases through dephosphorylation of an inhibitory tyrosine phosphorylation site at its carboxyl terminus (56–58). It is possible that Etk can also be activated by PTPD1 dephosphorylation. In this scenario, a phosphatase-inactive mutant of PTPD1 should fail to stimulate Etk kinase activity, leading to a decrease in tyrosine phosphorylation of Etk and PTPD1. To test this possibility, a phosphatase-inactive mutant (PTPD1 C1108S, with a conserved cysteine residue within the PTP domain changed to serine) was analyzed in coexpression with Etk. As shown in Fig. 4 (upper panel), this mutant could be detected in the immunoprecipitated complexes of Etk, and the amount of precipitated protein was very similar to that of the wild type, indicating that the phosphatase activity does not affect the Etk-PTPD1 interaction. Notably, this mutant conferred a higher phosphotyrosine content in both factors than did the wild type as detected by 4G10 antibody (middle panel). These findings imply that PTPD1 phosphatase activity is not involved in the induction of Etk kinase activity. Conversely, Etk may be negatively modulated by PTPD1 dephosphorylation.

We next asked whether the increased phosphorylation of Etk results in induction of its trans-phosphorylation activity. Etk was coexpressed with the empty expression vector, PTPD1, or PTPD1Δ(726–848) in 293T cells and then immunoprecipitated with anti-T7 antibody. An in vitro kinase assay was performed with the immunoprecipitated complexes of Etk and a peptide derived from the Btk SH3 sequence containing the major autophosphorylation site as an exogenous substrate. Etk immunoprecipitated from cells coexpressing PTPD1 showed significantly higher kinase activity than that immunoprecipitated from cells coexpressing the empty vector or PTPD1Δ(726–848) (Fig. 5). Consistent with above findings, these results indicate...
PTPD1 Induces Etk/Bmx Activity

Fig. 6. PTPD1 up-regulates the activity of Etk upon activation of Stat3.

A, COS cells were transfected with Stat3 expression vector together with various Etk and PTPD1 expression constructs as indicated. Cells were lysed, and the same amount of cell lysate from each fraction was subjected to immunoblotting using anti-phospho-Stat3 and anti-Stat3 antibodies (first and second panels, respectively). The protein expression level of PTPD1 and Etk was also determined by anti-HA and anti-T7 antibodies (third and fourth panels, respectively). WT, wild-type Etk; KQ, Etk K445Q. B, 293T cells were transfected with the indicated plasmids, and nuclear extracts were made for electrophoretic mobility shift assay 2 days after transfection (see “Experimental Procedures”). The Stat3-SIE complex is indicated. C, salivary Pa-4 cells were transfected with 0.6 μg of pLucTKS3 reporter in the presence (+) or absence (−) of 0.6 μg of Etk, 0.6 μg of Etk K445Q, and 0.7 μg of PTPD1 expression plasmid as indicated. After 30 h, cell lysates were collected, and the luciferase assay was performed (see “Experimental Procedures”). The reporter activity shown is the mean ± S.D., based on three independent transfection experiments. WB, Western blot.

that PTPD1 binds to Etk and stimulates Etk kinase activity.

Etk has been demonstrated to activate Stat1, Stat3, and Stat5 signaling pathways when coexpressed in COS cells (27). We next examined whether PTPD1 can enhance Etk activation in the Stat3 signaling pathway, including tyrosine phosphorylation at Tyr705, DNA binding, and transactivation. Coexpression of Etk and Stat3 in COS-7 cells increased the Tyr705 phosphorylation of Stat3 as detected by anti-phospho-Stat3 antibody (Fig. 6A, upper panel, lane 3). The phosphorylation of Stat3 was due to the kinase activity of Etk since Etk K445Q failed to confer the effect. Cells coexpressing PTPD1 and Etk conferred significantly higher tyrosine phosphorylation of Stat3, whereas cells coexpressing PTPD1Δ(726–848) did not significantly alter the content of phosphotyrosine. As controls for requirement of Etk in Stat3 activation, cells coexpressing PTPD1 with Etk K445Q or Stat3 with PTPD1 alone did not induce Stat3 phosphorylation (lanes 7 and 8). These results again suggest that PTPD1 stimulates the kinase activity of Etk, leading to the activation of Stat3. To further substantiate this finding, we performed a gel mobility shift assay with a Stat3-binding site (SIE). Coexpressing PTPD1 and Etk markedly induced the formation of the Stat3-SIE complex (Fig. 6B, lane 6), whereas Etk alone conferred a weak complex (lane 3). Furthermore, the activity of a Stat3-responsive reporter was examined. Consistent with the above findings, only cotransfection of PTPD1 with Etk led to a robust activation of the reporter (Fig. 6C).

PTPD1 Potentiates Etk, but Not Jak2, in Activating the Stat3 Signaling Pathway—To demonstrate the specific effect of PTPD1 on Etk-Stat3 activation, we used the Jak2 kinase in coexpression with PTPD1. As illustrated in Fig. 7A, overexpressing Jak2 alone was sufficient to increase tyrosine phosphorylation of Stat3 (lane 4). Coexpression of PTPD1 with Jak2 resulted in no significant change in Stat3 tyrosine phosphorylation. In control experiments, PTPD1 could still induce Stat3 phosphorylation through Etk, whereas overexpressed Etk alone was not sufficient to give detectable phosphorylation in 293T cells. The protein expression level of Stat3 and PTPD1 in the cells coexpressing Jak2 was examined and shown to be very similar, if not identical, to that in the cells coexpressing Etk. These findings were further confirmed by the Stat3 reporter gene assay. A dosage-dependent effect of PTPD1 on Etk (but not Jak2)-induced activation of Stat3 is shown in Fig. 7B. Taken together, our results firmly demonstrate the specificity of PTPD1 in Etk-Stat3 activation.

Specificity of PTPD1 in Activation of Tec Family Kinases—The amino acid residues of the PH domain are highly conserved in the Tec family members. Our finding that the PH domain of Etk is critical for PTPD1 binding and regulation raises the possibility that PTPD1 can also regulate the activity of other members of the Tec kinase family. To test this possibility, Btk and Tec kinases were assayed for the induction of tyrosine phosphorylation by PTPD1. The overexpressed kinases were immunoprecipitated and subjected to Western analysis with 4G10 antibody. Like Etk, Tec could be regulated by PTPD1 as demonstrated by the increased tyrosine phosphorylation of Tec and associated PTPD1 (Fig. 8, upper panel, lanes 7 and 9), despite the fact that Tec had a higher basal phosphotyrosine content. In contrast, Btk could not be activated by PTPD1. As a positive control for Btk activation, a constitutively active form of c-Src Y527F was utilized to stimulate Btk phosphorylation (lane 5). These results indicate that PTPD1 can also bind to Tec kinase and regulate its activity.

DISCUSSION

Protein-tyrosine phosphatases have the potential to exert a considerable influence on tyrosine phosphorylation-dependent signaling pathways, both augmenting and antagonizing the function of protein-tyrosine kinases. In this study, we have shown that PTPD1 regulates Etk activity and that the regulation is directly mediated by the Etk-PTPD1 protein interaction. Several lines of evidence support this conclusion. First, wild-type PTPD1 and Etk can form a complex in vitro and in vivo. The interaction leads to activation of the kinase activity of Etk as demonstrated by increasing the phosphotyrosine content of Etk, PTPD1, and Stat3 as well as trans-phosphorylation of an
PTPD1 Induces Etk/Bmx Activity

exogenous substrate. Second, deletion of the PTPD1 fragment containing residues 726–848, which is essential for Etk binding, resulted in loss of induction of Etk activity as demonstrated by no increased tyrosine phosphorylation of both Etk and Stat3 as well as no significant change in kinase activity on an exogenous substrate. Third, the PH domain deletion mutant caused no interaction with PTPD1 and failed to confer increased tyrosine phosphorylation of Etk and Stat3 (data not shown). Fourth, PTPD1 markedly enhanced Stat3 activation through Etk, but not Jak2. Although the evaluation of the physiological relevance of this association is still in progress, these findings strongly suggest that PTPD1 can modulate the function of Etk in tyrosine phosphorylation-dependent signaling pathways.

Previous studies of PTPD1 indicated that Src kinase can associate with and phosphorylate PTPD1 (54). These findings led us to consider the possibility that phosphorylation of Etk induced by PTPD1 was due to Src kinase recruited by PTPD1. To address this possibility, we overexpressed Csk, a tyrosine kinase that can inhibit the kinase activity of Src family kinases (29). In support of this thesis, it was previously shown that Galphaq and Galpha12, which bind to the TH-SH3 and PH-TH domains of Btk, respectively, are strong activators of Btk (32–34), and our own work revealed that removal of the PH domain constitutively activates Etk (7). Thus, a common regulatory mechanism of the Tec family kinases is through competitive binding of a ligand (lipid or protein) that disrupts the intramolecular folding, giving access to the Src-like kinase to phosphorylate the catalytic domain of Tec, hence activating the kinase.

Our findings that PTPD1 can selectively activate Etk and Tec, but not Btk, also indicate the specificity in this type of ligand-kinase interaction. Sequence alignment of the PH domains of Btk, Tec, and Etk shows considerable sequence similarity and identity (59). The sequences are more variable in their N-terminal halves, and there are no insertions or deletions in the C-terminal halves including the Btk motif. The scaffold-
PTPD1 Induces Etk/Bmx Activity

Phosphatase D1 (PTPD1) has recently been shown to induce Etk/Bmx activity through protein-protein interaction. The feasibility of this interaction remains to be determined.

The ectodomain of PTPD1 is homologous to cytoskeleton-associated proteins of the band 4.1 superfamily, including band 4.1, ezrin, radixin, moesin, and merlin. The presence of such a targeting domain implies that PTPD1 may be also directed to cytoskeleton-membrane interfaces and that the subcellular localization of PTPD1 may be determined. Thus, it is possible that the Etk-PTPD1 interaction and increased tyrosine phosphorylation of both factors may have some effect on their subcellular localization. Furthermore, Dorner et al. (74) has recently demonstrated that KIF1C, a new kinesin-like protein, can bind to the ezrin-like domain of PTPD1. The KIF1C protein is involved in vesicle transport from the Golgi apparatus to the endoplasmic reticulum. Whether the Etk-PTPD1 interaction affects the function of KIF1C remains to be addressed. Another possible effect of the Etk-PTPD1 interaction is to alter the phosphatase activity of PTPD1. Additional studies are required to further explore the biological effects of PTPD1.

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PTPD1 Induces Etk/Bmx Activity

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