Mutual Regulation of Protein-tyrosine Phosphatase 20 and Protein-tyrosine Kinase Tec Activities by Tyrosine Phosphorylation and Dephosphorylation*

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PTP20, also known as HSCF/protein-tyrosine phosphatase K1/fetal liver phosphatase 1/brain-derived phosphatase 1, is a cytosolic protein-tyrosine phosphatase with unknown biological relevance. We have identified that the nonreceptor protein-tyrosine kinase Tec-phosphorylated PTP20 on tyrosines and coimmunoprecipitated with the phosphatase in a phosphotyrosine-dependent manner. The interaction between the two proteins involved the Tec SH2 domain and the C-terminal tyrosine residues Tyr-281, Tyr-303, Tyr-354, and Tyr-381 of PTP20, which were also necessary for tyrosine phosphorylation/dephosphorylation. Association between endogenous PTP20 and also tyrosine phosphorylation-dependent negative feedback mechanism. It appears to play a negative role in Tec-mediated signal.

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‡‡The abbreviations used are: PTP, protein-tyrosine phosphatase; HSCF, hematopoietic stem cell fraction; PSTPIP, proline, serine, threonine phosphatase-interacting protein; PTK, protein-tyrosine kinase; BCR, B cell receptor; SH2, Src homology 2; SH3, Src homology 3; HA, hemagglutinin; GST, glutathione S-transferase; WT, wild type; ECL, enhanced chemiluminescence; PH, pleckstrin homology; TH, Tec homology; POV, pervanadate.

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becomes tyrosine-phosphorylated by constitutively active forms of Lck and v-Src kinases in transfected cells (8, 11) even though the physiological relevance of tyrosine phosphorylation on PTP20 remains unclear.

In this study we addressed the question of PTP20 regulation with special emphasis on the relevance of tyrosine phosphorylation and its biological impact. Through co-expression with various PTKs we found that Tec kinase strongly tyrosine-phosphorylated the catalytically inactive form of PTP20 and that Tec physically interacted with PTP20 in a tyrosine phosphorylation-dependent manner in transfected COS7 cells. Further analyses with a variety of mutants of PTP20 and Tec revealed that C-terminal tyrosine residues of PTP20 and the Tec SH2 domain were necessary in the regulation of respective state of phosphorylation. Ectopic expression of PTP20 in human immature Ramos B cells resulted in suppression of B-cell receptor-induced c-fos promoter activity. Moreover, we determined that tyrosine 281 of PTP20 plays a role in the dephosphorylation activity of PTP20 against both Tec and PTP20 itself. Our findings suggest a negative feedback mechanism that mutually controls the tyrosine phosphorylation of Tec and PTP20 and regulates Tec activity and B cell receptor (BCR) signaling.

EXPERIMENTAL PROCEDURES

Reagents—Antibodies to hemagglutinin (HA) epitope (Y-11), phospho-tyrosine (PTP199), glutathione S-transferase (GST) (Z-6), Src (SRC2), Lck (2102), Jak2 (M-126), Jak3 (C-21), Csk (C-20), and Zap70 (LR) were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Antibodies to Tec, Itk, Btk, and Bmx were as described previously. Antibody to PTP20 was prepared by immunizing rabbits with a synthetic peptide corresponding to the C-terminal peptide of PTP20 (MSRQSDLVRSFLEQQEAP). A synthetic peptide corresponding to the cysteine residue was added to the C-terminus, causing the antibody to react with recombinant PTP20 (14) Anti-human JgM antibody (IgG1) was purchased from Dako.

Plasmid Construction—With the use of the dual luciferase assay system (Promega, Madison, WI). PTP20 was abundantly expressed in spleen, suggesting that PTP20 was involved in PTP20 regulation by tyrosine phosphorylation. To examine this possibility HA-tagged PTP20 was co-expressed with various cytosolic PTKs including Src and Lck in COS7 cells. We used a catalytically inactive form of PTP20 for this experiment because autodephosphorylation activity of PTP20 has been previously shown to be low. As shown in Fig. 1A, Src and Lck co-immunoprecipitated with PTP20 in transfected cells. Western blot analysis revealed that PTP20 was immunoprecipitated with antibodies to Src and Lck as well as HA epitope and the immune complexes were detected with anti-HA antibody. The western blot analysis again confirmed the results of co-immunoprecipitation experiment because autodephosphorylation activity of PTP20 was observed in the case of Src but not Lck.

Cell Lysis, Immunoprecipitation, GST Pull-down, and Western Blotting—The transfected cells were lysed with lysis buffer containing 50 mM Tris–HCl (pH 7.5), 5 mM EDTA, 150 mM NaCl, 10 mM sodium phosphate, 10 mM sodium fluoride, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml leupeptin. Lysates were subjected to immunoblotting, immunoprecipitation with the indicated antibodies plus protein G- or Protein A-Sepharose beads (Amersham Bioscience), or precipitation with GSH-Sepharose beads (Amersham Bioscience). Proteins in the immunoprecipitates and precipitates were further analyzed by immunoblotting with the indicated antibodies. The protein bands were visualized with an enhanced chemiluminescence (ECL) detection kit (Amersham Bioscience) and light capture system (ECL). The transfected cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. Expression plasmids were transfected into the cells with Lipofectamine (Life Technologies, Inc.) as described previously. The transfected cells were lysed with lysis buffer containing 50 mM Tris–HCl (pH 7.5), 5 mM EDTA, 150 mM NaCl, 10 mM sodium phosphate, 10 mM sodium fluoride, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml leupeptin. Lysates were subjected to immunoblotting, immunoprecipitation with the indicated antibodies plus protein G- or Protein A-Sepharose beads (Amersham Bioscience), or precipitation with GSH-Sepharose beads (Amersham Bioscience) after treatment with protease inhibitors. The protein bands were visualized with an enhanced chemiluminescence (ECL) detection kit (Amersham Bioscience) and light capture system (ECL).
Phosphotyrosine-dependent Interaction between PTP20 and Tec—Tec is composed of several distinct domains including pleckstrin homology (PH), Tec homology (TH), SH3, SH2, and kinase (KD) domains (Fig. 3, panel A). All of these domains are necessary for full function of Tec under physiological conditions (15, 16). To examine which domains are involved in interaction with PTP20, Tec mutants each lacking one of the domains were co-transfected with the catalytically inactive form of PTP20 into COS7 cells. A kinase mutant as well as two mutants (Y187F and Y518F) where tyrosine residues were replaced by phenylalanines were also included. Cells were lysed, and PTP20 was immunoprecipitated followed by immunoblotting with anti-phosphotyrosine antibody. PTP20 was tyrosine-phosphorylated by the Y187F mutant as well as mutants lacking PH, TH, and SH3 domains to a similar extent as compared with Tec WT (Fig. 3, panel B). As expected, the Y518F mutant, which is missing the autophosphorylation site for Tec activation, and the inactive mutant of a kinase mutant could not tyrosine phosphorylate PTP20. Interestingly, the ΔPH mutant could tyrosine phosphorylate PTP20 but was not co-immunoprecipitated with PTP20. Most strikingly, the ΔSH2 mutant could not tyrosine phosphorylate PTP20 and was not co-immunoprecipitated with PTP20. When a membrane on which aliquots of total cell lysates were blotted was probed with anti-phosphotyrosine antibody, it was revealed that co-expression of the Tec ΔSH2 mutant and PTP20 resulted in no tyrosine phosphorylation on both molecules and that the Tec ΔPH mutant tyrosine-phosphorylated (Fig. 3, panel C). Tec SH2 domain-dependent interaction with PTP20 was further investigated by co-transfecting the PTP20 C/S mutant with plasmids encoding GST fusion proteins of Tec domains in the presence or absence of Tec into COS7 cells. Cell lysates were subjected to pull-down experiments with GSH-Sepharose beads. Precipitates were separated by SDS-PAGE and subsequently probed with an anti-phosphotyrosine antibody, and the TEC domain of full-length Tec co-expression proteins (GST or GST-TEC) were precipitated with PTP20 to any of the Tec domains (Fig. 3, panel D). In contrast, the non-phosphorylatable mutant of the PH domain of Tec, the inactive mutant of a kinase mutant could not tyrosine phosphorylate PTP20 and was not co-immunoprecipitated with PTP20. Most strikingly, the ΔSH2 mutant could tyrosine phosphorylate PTP20 but was not co-immunoprecipitated with PTP20. These results suggest that PTP20 is a substrate of Tec and that Tec is also a substrate of PTP20.

To identify the binding site(s) for Tec in PTP20, we used GST pull-down experiments with GSH-Sepharose beads. Precipitates were separated by SDS-PAGE and probed with the indicated antibodies to confirm substantial expression of each PTK.

We focused our attention on the tyrosine residues Tyr-281, Tyr-303, Tyr-354, and Tyr-381 located in the PTP20 sequence, and all the residues are perfectly conserved among human and mouse orthologs (Fig. 4). There are 13 tyrosine residues (Tyr-62, Tyr-68, Tyr-86, Tyr-101, Tyr-144, Tyr-192, Tyr-244, Tyr-281, Tyr-285, Tyr-303, Tyr-354, Tyr-381, Tyr-419) in the PTP20 sequence, and all the residues are perfectly conserved among human and mouse orthologs (Fig. 4). We focused our attention on the tyrosine residues Tyr-281, Tyr-285, Tyr-303, Tyr-354, Tyr-381, and Tyr-419 located in the C-terminal PEST domain of PTP20, and 6 residues were individually mutated.

First, the mutants were tested for the extent of tyrosine phosphorylation by Tec in transfected COS7 cells. Total cell lysates were subjected to anti-phosphotyrosine blotting. Fig. 5, panel A, demonstrates that the PTP20 mutants (Y281F, Y303F, Y354F, Y381F) in which Tyr-281, Tyr-303, Tyr-354, and Tyr-381 were individually mutated exhibited dramatic reduction in tyrosine phosphorylation levels, whereas no apparent reduction for Y285F and Y419F was observed. Combinatorial mutation of Tyr-281, Tyr-303, Tyr-354, and Tyr-381 totally abolished tyrosine phosphorylation of PTP20. In keeping with these data, anti-phosphotyrosine blotting also demonstrated that tyrosine phosphorylation of Tec was concomitantly reduced. This observation was further extended by GST pull-down experiments using the Tec SH2 domain. COS7 cells were then transfected with PTP20 YF variants together with Tec and Tec-Tec SH2, as outlined in Fig. 3, panel C. Mutation of either Tyr-281, Tyr-303, Tyr-354, or Tyr-381 of PTP20 resulted in reduced binding capacity of PTP20 to the Tec SH2 domain, and again, such binding was completely abrogated by substituting all the tyrosine residues (Fig. 5, panel B). Together these data clearly indicate that four tyrosine residues in the C-ter-
minal non-catalytic region of PTP20 are involved in not only binding to the Tec SH2 domain but also in the phosphorylation and subsequent activation of Tec.

We asked whether the C-terminal non-catalytic region of PTP20 was enough for phosphorylation and activation of Tec. To this end, PTP20 deletion mutants lacking either an N-terminal catalytic or a C-terminal non-catalytic segment were made, but the resultant constructs could not be expressed in COS7 cells, although comparable amounts of transcripts were detected (data not shown). To solve this problem, the N-terminal PTP domain and the C-terminal PEST domain were inserted into pEBG vector and were expressed as GST fusion proteins in COS7 cells. These pEBG plasmids encoding the PTP domain and full length of PTP20 C/S mutant and the PTP domain and C-terminal PEST domain were in-
dected (data not shown). To solve this problem, the N-termi-
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Negative Regulatory Roles of PTP20 in BCR Signaling—All the experiments documented above were conducted in transfect- ed COS7 cells. To demonstrate a physiological relevance of the PTP20-Tec interaction, evidence of such an association in non-transfected cells was required. To this end we selected human Ramos immature B cells, because it has been reported that they express relatively high amounts of endogenous Tec (21). As shown above, interaction of PTP20 with Tec is medi- ated by tyrosine phosphorylation of PTP20, and PTP20 has autodephosphorylation activity, implying that it would be dif-

Although upstream regulators such as cytokine receptors, lymphocyte surface antigens, G protein-coupled receptors, re-
ceptor type PTKs, or integrins for Tec in blood cells including
Ramos B cells have been relatively well investigated (13, 20,
22–26), only limited information regarding downstream regu-
laters of Tec has been available so far. If the data obtained in
transfected COS7 cells are true, PTP20 would be thought to
play a negative regulatory role in Tec-mediated signaling. To
examine this, either the PTP20 WT, the inactive C/S mutant, or
another form of catalytically inactive mutant D/A was tran-

Tyrosine Phosphorylation of PTP20 by Tec Modulates Its Catalytic Activity against Tec as Well as Itself—We demon-
strated that specific tyrosine residues Tyr-281, Tyr-303, Tyr-
354, and Tyr-381 of PTP20 could be phosphorylated by Tec and
served as Tec binding sites (Fig. 5). To further investigate
physiological relevance of PTP20 tyrosine phosphorylation,
substitution of the tyrosine residues with phenylalanine in
PTP20 WT was performed. The YF mutants of HA-PTP20 WT

Fig. 2. Tyrosine phosphorylation-dependent interaction of PTP20 with Tec. Tec cDNA was transiently introduced into COS7 cells together with either empty vector (mock), HA-PTP20 WT, or C/S and lysed. PTP20 or Tec was immunoprecipitated either with anti-HA (left panels) or anti-Tec (right panels) antibody, respectively. The immunoprecipitates (IP) were separated by SDS-PAGE followed by immunoblotting (WB) sequentially with the indicated antibodies. The bands corresponding to Tec and PTP20 are indicated by arrows. In either case expression of Tec or HA-PTP20 was confirmed using aliquots of total cell lysates (TCL) by immunoblotting (lowest panels). αY, anti-phosphotyrosine antibody.
Fig. 3. Tec SH2 domain is essential for both tyrosine phosphorylation of PTP20 and association of Tec with PTP20. A, schematic organization of mouse Tec into PH, TH, SH3, SH2, and kinase (KD) domains. B, COS7 cells were transiently transfected with either empty vector (mock) or HA-PTP20 C/S together with the indicated Tec mutants. Cells were lysed, and HA-PTP20 was immunoprecipitated (IP) followed by immunoblotting (IB) with anti-phosphotyrosine antibody (PY99 (pY)). The same membrane was sequentially reprobed with anti-Tec and anti-HA antibodies after stripping. C, aliquots of the total cell lysates (TCL) were separated by SDS-PAGE followed by immunoblotting with indicated antibodies. D, COS7 cells were transiently transfected with pEBG empty vector (GST) or bearing each of Tec domains (PH, TH, SH3, SH2, and KD) in the absence or presence of Tec plasmid. GST fusion proteins precipitated by GSH-Sepharose beads were analyzed by immunoblot analysis with anti-phosphotyrosine (pY) antibody. Expression of PTP20 and Tec was confirmed using aliquots of total cell lysates (TCL) by immunoblotting as indicated.
were co-transfected with Tec and the PTP20 C/S mutant without epitope tagging, and effects on the extent of tyrosine phosphorylation on Tec were analyzed by anti-phosphotyrosine blotting. As shown in Fig. 9A, substitution of Tyr-281 with phenylalanine (Y281F) resulted in dramatic loss of PTP20 dephosphorylation activity against Tec. On the other hand, Tec could be dephosphorylated by Y303F, Y354F, and Y381F to nearly the same extent by PTP20 WT. The PTP20 Y281F/Y303F/Y354/F381F mutant in which 4 tyrosine residues were substituted by phenylalanine also exhibited apparently no dephosphorylation activity against Tec. Equivalent expression of HA-PTP20 was confirmed by immunoblotting (lowest panel).

Next, the autodephosphorylation activity of the YF mutants of HA-PTP20 WT was assessed by co-transfecting Tec and PEST encoding the GST-PTP20 PEST domain into COS7 cells, as GST-PTP20 PEST alone became tyrosine-phosphorylated in the presence of Tec (Fig. 6). Cells were lysed and GST-PTP20 PEST was precipitated with GSH-Sepharose beads followed by anti-phosphotyrosine blotting. Again, PTP20 Y281F as well as PTP20 Y281F/Y303F/Y354/F381F showed no dephosphorylation activity against GST-PTP20 PEST, whereas PTP20 Y303F, Y354F, and Y381F as well as PTP20 WT could dephosphorylate GST-PTP20 PEST (Fig. 9B). These YF mutants also were transfected into Ramos B cells, and c-fos promoter activity was assayed after BCR ligation. Ectopic expression of PTP20 Y281F and Y281F/Y303F/Y354/F381F mutants still inhibited c-fos promoter activity (about 50%, relative to mock transfectants), but the extent was significantly lower than that of WT as well as other YF mutants. These results strongly suggest that phosphorylation of Tyr-281 on PTP20 is essential for expression of catalytic activity against not only Tec but also PTP20 itself in transfected COS7 cells as well as in Ramos B cells, although other tyrosine residues, Tyr-303, Tyr-354, and Tyr-381, are also phosphorylated by Tec.

DISCUSSION

Many signaling pathways triggered by PTKs can be potentially modulated by PTPs in a negative or positive manner under cellular context. In some cases phosphorylation on the tyrosine residues of PTPs themselves can modulate their catalytic activities. For example, SH2 domain-containing PTP SHP-2 is tyrosine-phosphorylated upon stimulation by a variety of growth factors (27–29) and cytokines (30–35). Once SHP-2 becomes tyrosine-phosphorylated, their catalytic activity might be increased and modulated its own tyrosine phosphorylation level by autodephosphorylation activity (36, 37). It also has been reported that tyrosine phosphorylation of PTP1B upon insulin and epidermal growth factor treatment causes reduction in its catalytic activity, thereby enhancing apparent insulin receptor- and epidermal growth factor receptor-mediated signaling pathways (38, 39). Thus, tyrosine phosphorylation of PTPs appeared to be critical for the regulation of their biological functions. Among the PEST family PTPs, PTP20 is an only member that gets phosphorylated on tyrosine residues, whereas no tyrosine phosphorylation of other members, PTP-PEST and PTP-
Mutual Regulation of PTP20 and Tec

Tyrosine phosphorylation of Tec and association with Tec SH2 domain. A, HA-PTP20 C/S or its YF (tyrosine to phenylalanine substitution) mutants as indicated were co-transfected into COS7 cells with Tec. Aliquots of total cell lysates (TCL) were immunoblotted (WB) with anti-phosphotyrosine (αpY) antibody. The same membrane was sequentially reprobed with anti-Tec and -HA antibodies. B, COS7 cells were co-transfected with expression plasmids for HA-PTP20 C/S or its YF mutants, Tec, and GST-Tec-SH2 domain. Cells were lysed, and GST-Tec-SH2 domain was precipitated with GSH-Sepharose beads followed by immunoblot analysis by sequential probing with anti-phosphotyrosine, anti-HA, and anti-GST antibodies. Expression of nearly the same amounts of PTP20 was confirmed by immunoblotting of aliquots of total cell lysates with anti-HA antibody.

Fig. 5. Specific tyrosine residues of PTP20 are necessary for tyrosine phosphorylation of Tec and association with Tec SH2 domain. A, HA-PTP20 C/S or its YF (tyrosine to phenylalanine substitution) mutants as indicated were co-transfected into COS7 cells with Tec. Aliquots of total cell lysates (TCL) were immunoblotted (WB) with anti-phosphotyrosine (αpY) antibody. The same membrane was sequentially reprobed with anti-Tec and -HA antibodies. B, COS7 cells were co-transfected with expression plasmids for HA-PTP20 C/S or its YF mutants, Tec, and GST-Tec-SH2 domain. Cells were lysed, and GST-Tec-SH2 domain was precipitated with GSH-Sepharose beads followed by immunoblot analysis by sequential probing with anti-phosphotyrosine, anti-HA, and anti-GST antibodies. Expression of nearly the same amounts of PTP20 was confirmed by immunoblotting of aliquots of total cell lysates with anti-HA antibody.

PEP, has been reported. In the present study, we clearly demonstrated that PTP20 was tyrosine-phosphorylated by a cytosolic Tec kinase. As previously reported for phosphorylation of PTP20 by constitutively active Src family kinases (8, 11), the catalytically inactive form of PTP20 was found to be tyrosine-phosphorylated to a greater extent by Tec, whereas apparently no phosphorylation on PTP20 WT was obvious, possibly due to its autodephosphorylation activity. Src and Lck indeed tyrosine-phosphorylated PTP20, but the extent of tyrosine phosphorylation of PTP20 by Tec was shown to be the greatest (Fig. 1). Moreover, related Itk did tyrosine-phosphorylate PTP20 to a lesser extent, but Btk and Bmx did not (Fig. 1). These results suggest that Tec kinase tyrosine phosphorylates PTP20 more specifically and preferentially than Src family kinases and its related kinases do.

Without ectopic PTP20 expression, tyrosine phosphorylation of Tec kinase was not detected in transfected COS7 cells (Fig. 2). Although co-expression of PTP20 WT did not induce tyrosine phosphorylation of Tec, the catalytically inactive C/S variant of PTP20 caused tyrosine phosphorylation of Tec and co-immunoprecipitated with Tec. These results strongly suggest that a dominant-negative effect of PTP20 C/S expression on Tec tyrosine phosphorylation seems to be unlikely and, rather, that Tec was possibly autophosphorylated and further activated by interacting with PTP20 and then was immediately dephosphorylated and deactivated by PTP20, which might also be activated through interaction with Tec in a tyrosine phosphorylation-dependent manner. A deletion of the Tec SH2 domain abrogated tyrosine phosphorylation of Tec as well as PTP20 and association between Tec and PTP20 (Fig. 3). Likewise, substitution of individual tyrosine residues Tyr-281, Tyr-303, Tyr-354, and Tyr-381 with phenylalanines of PTP20 reduced not only tyrosine phosphorylation of Tec and PTP20 itself but also association of PTP20 with the Tec SH2 domain (Fig. 5). Substitution of all the four tyrosine residues (Fig. 5) as well as a deletion of the catalytically inactive C-terminal segment (Fig. 6) completely abrogated tyrosine phosphorylation of Tec (Fig. 3), and its substrate is often low. Actually, the PTP domain of the catalytically inactive PTP20 alone could not capture a potential substrate Tec kinase (Fig. 6). A further enhancement of the specificity is achieved by protein-protein targeting; the Tec SH2 domain and phosphorylated tyrosine residues on PTP20 could enhance the interaction between the two molecules. In Ramos B cells, we could detect tyrosine phosphorylation-dependent interaction between PTP20 and Tec only when cells were treated with pervanadate (Fig. 7). In this case, however, apparent binding might have resulted from a sole interaction of phosphorylated tyrosines of PTP20 C-terminal with the Tec SH2 domain and, therefore, underestimated because vanadate can get into the catalytic pocket of PTP20 reversibly and inhibit interaction between the PTP domain segment of PTP20 and tyrosine-phosphorylated Tec kinase. Upon physiological stimulation both PTP20 catalytic domain-Tec phosphotyrosines) and PTP20 phosphotyrosine-Tec SH2 domain bindings could play an essential role.

Most interestingly, tyrosine phosphorylation of PTP20 appears to regulate its catalytic activity against Tec and PTP20 itself. Among the tyrosine residues phosphorylated by Tec kinase, tyrosine 281 might be critical for dephosphorylation activity of PTP20 in transfected COS7 cells as well as in Ramos B cells (Fig. 9). In the case of ectopic expression in COS7 cells, substitution of the Tyr-281 nearly abolished dephosphorylation activity against both PTP20 and Tec (Fig. 9, A and B). On the other hand Y281F as well as Y281F/Y303F/Y354F/Y381F mutants exhibited reduced, but still ~50% dephosphorylation activity as compared with mock transfectants when expressed in

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Ramos B cells (Fig. 9C), suggesting that other direct or indirect mechanisms to regulate PTP20 activity are involved in dephosphorylation and deactivation of Tec in the cells. However, we cannot exclude the possibility that phosphorylation on serine and threonine residues rich in the C-terminal region of PTP20 might affect catalytic activity of PTP20, as PTP20 can be regulated under the control of follicle-stimulating hormone in rat ovarian granulosa cells, where no tyrosine phosphorylation on PTP20 was observed (14).

It has been reported that constitutively active Lck phosphorylates tyrosine residues 354 and 381 on PTP20, which in turn recognized by the Csk SH2 domain (11). In that report it was also documented that mutation of both the tyrosine residues leads to decreased interaction with Tec.

Fig. 6. Both PTP catalytic and PEST domains of PTP20 are involved in maximal phosphorylation of Tec and association with Tec. Ramos cells were co-transfected with either empty pEBG vector (−) or a non-catalytic PEST domain of PTP20 (PEST). A, aliquots of total cell lysates (TCL), were subjected to immunoblotting with anti-phosphotyrosine antibody (αpY, upper panel). The same membrane was sequentially probed with anti-Tec and anti-GST antibodies. The bands corresponding to Tec and PTP20 are indicated by arrows.

Fig. 7. Tyrosine phosphorylation-dependent interaction of endogenous PTP20 with endogenous Tec in Ramos B cells. Ramos cells were treated with 0.1 mM POV for 15 min at 37 °C, lysed, and subjected to immunoprecipitation with either anti-phosphotyrosine (αpY) or anti-Tec antibody. The immunoprecipitates (IP) were immunoblotted (WB) by anti-phosphotyrosine antibody. The same membranes were sequentially reprobed with anti-PTP20 and -Tec antibodies. The bands corresponding to Tec and PTP20 are indicated by arrowheads.

Fig. 8. Negative role of PTP20 in BCR signaling. Ramos cells (1 × 10⁷) were subjected to electroporation with 2 μg of the pfos/luc reporter plasmid together with 10 μg of pcDNA3 vector (mock) or bearing PTP20 WT, C/S, or D/A mutant. Five hours after transfection, cells were incubated for an additional 5 h in the absence (open bars) or presence (closed bars) of anti-IgM (ab′) (10 μg/ml). Cells lysates were then assayed for luciferase activity. Data are expressed as mean ± S.D. of triplicate determinations.
Fig. 9. Tyrosine 281 is critical for in vivo phosphatase activity of PTP20.

**A**, COS7 cells were co-transfected with Tec, PTP20 C/S, and HA-PTP20 WT or its YF mutants. HA-PTP20 C/S was also included as a negative control. Cells were lysed, and Tec was immunoprecipitated with anti-Tec antibody. The immunoprecipitates (IP) were separated by SDS-PAGE followed by immunoblotting (WB) with indicated antibodies. Expression of HA-PTP20 was confirmed using aliquots of total cell lysates (TCL) with anti-HA antibody, αY, anti-phosphotyrosine antibody. 

**B**, COS7 cells were transfected as above, but PEST-encoding GST-PTP20 PEST domain (GST-PEST) in place of PTP20 C/S was included. Cell lysates were subjected to precipitation with GSH-Sepharose beads and immunoblotted with the indicated antibodies. Expression of HA-PTP20 was confirmed using aliquots of total cell lysates (TCL) with anti-HA antibody. 

**C**, Ramos cells were transfected by electroporation with 2 μg of pfos/luc reporter plasmid together with 10 μg of pcDNA3 vector (mock) or expressing PTP20 WT or its YF mutant and processed as described in legend to Fig. 8.
dues on PTP20 caused no changes in catalytic activity by in vitro phosphatase assay. We have also showed that PTP20 was tyrosine-phosphorylated by Lck and Src and was associated with the PTKs (Fig. 2). However, neither the SH2 nor the SH3 domain of Lck was shown to be involved in the association with PTP20 (data not shown). Recently, another cytosolic protein-tyrosine kinase c-Abl also was shown to phosphorylate PTP20 and in turn to be dephosphorylated by PTP20 (10). Although PTP20-Tec and PTP20-cAbl interactions seem to be analogous, association between PTP20 and c-Abl is indirect, and PSTPIP, which is also a substrate of PTP20, instead serves as an adapter by bridging PTP20 to c-Abl. In contrast, association between PTP20 and Tec kinase seems to be direct, and involvement of adaptor molecules such as PSTPIPs is unlikely because the Tec SH2 domain alone could capture tyrosine-phosphorylated PTP20 (Fig. 3D). These imply that PTP20 might be differentially tyrosine-phosphorylated by Lck, Tec, and c-Abl kinases depending on cellular context.

The Tec kinase was initially isolated from mouse liver (40) and was subsequently shown to be expressed in many tissues, including spleen, lung, brain, and kidney (41). Four Tec-related PTKs, including Btk (42, 43), Itk (also known as Emt or Tsk) (44–46), Bmx (47), and Txk (or Rlk) (48, 49), have also been molecularly cloned. Tec and the related kinases can be activated by cytokine receptors, lymphocyte surface antigens, G protein-coupled receptors, receptor type PTKs, or integrins (13, 20, 22–26). However, little is known about how the inactivation of Tec kinase is achieved. In this study, we have showed that PTP20 is a potential negative regulator in Tec-mediated signaling pathway and that the Tec SH2 domain is essential for negative regulation by PTP20. Itk, another member of Tec family, might also be regulated by PTP20 in T cells in a similar fashion, whereas Btk and Bmx seem not to interact with PTP20 (Fig. 1). Recently, the Tec SH2 domain has been shown to bind to Dok-1, which is tyrosine-phosphorylated by Tec, causing inhibition of BCR-mediated c-fos promoter activation (18). Another publication has demonstrated that a docking protein, BRDG1, binds to the Tec SH2 domain and acts downstream of Tec in a positive fashion in BCR signaling (50). Thus, the Tec SH2 domain might differentially participate in BCR signaling in a positive or negative way.

PTP D1, which comprises another subfamily of cytosolic PTPs, is shown to be a potential regulator and effector for not only Bmx/Etk kinase but also Tec kinase (51). The PH but not SH2 domain of Bmx/Etk is involved in the interaction with the central portion (residues 726–848) of PTP D1, and such binding is phosphotyrosine-independent, unlike PTP20-Tec interaction. Interaction between Bmx/Etk and PTP D1 stimulates the kinase activity of Bmx/Etk, resulting in an increased phosphotyrosine content in both proteins. Although it is obvious that PTP D1 is a substrate of Bmx/Etk and Tec, PTP D1 appears not to dephosphorylate the kinases. Rather, PTP D1 is a positive regulator in Bmx/Etk- and Tec-mediated signaling pathway leading to STAT3 activation. By co-transfection experiments, we observed that PTP36, which belongs to the same PTP subfamily as PTPD1, was tyrosine-phosphorylated by Tec kinase (data not shown). Thus, Tec-mediated signaling could be negatively or positively regulated by interacting with PTPs.

In conclusion, PTP20 appears to play a negative role in the...
Mutual Regulation of PTP20 and Tec

Tec-mediated, in particular in BCR, signaling pathways and the tyrosine phosphorylation-dependent interaction between Tec and PTP20 might form a negative feedback loop. To our knowledge this is the first report demonstrating that tyrosine phosphorylation-dependent interaction between FPT and PTP is relevant for their mutual state in some cellular context.

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