We have examined the \textit{in vitro} activity of receptor-like protein-tyrosine phosphatase \( \alpha \) (PTP\( \alpha \)) toward p59\( ^{fyn} \), a widely expressed Src family kinase. In a coexpression system, PTP\( \alpha \) effected a dose-dependent tyrosine dephosphorylation and activation of p59\( ^{fyn} \), where maximal dephosphorylation correlated with a 5-fold increase in kinase activity. PTP\( \alpha \) expression resulted in increased accessibility of the p59\( ^{fyn} \) SH2 domain, consistent with a PTP\( \alpha \)-mediated dephosphorylation of the regulatory C-terminal tyrosine residue of p59\( ^{fyn} \). No p59\( ^{fyn} \) dephosphorylation was observed with an enzymatically inactive mutant form of PTP\( \alpha \) or with another receptor-like PTP, CD45. Many enzyme-linked receptors are complexed with their substrates, and we examined whether PTP\( \alpha \) and p59\( ^{fyn} \) underwent association. Reciprocal immunoprecipitations and assays detected p59\( ^{fyn} \) and an appropriate kinase activity in PTP\( \alpha \) immunoprecipitates and PTP\( \alpha \) and PTP activity in p59\( ^{fyn} \) immunoprecipitates. No association between CD45 and p59\( ^{fyn} \) was detected in similar experiments. The PTP\( \alpha \)-mediated activation of p59\( ^{fyn} \) is not prerequisite for association with wild-type and inactive mutant PTP\( \alpha \) bound equally well to p59\( ^{fyn} \). Endogenous PTP\( \alpha \) and p59\( ^{fyn} \) were also found in association in mouse brain. Together, these results demonstrate a physical and functional interaction of PTP\( \alpha \) and p59\( ^{fyn} \) that may be of importance in PTP\( \alpha \)-initiated signaling events.

Members of the Src family of tyrosine kinases have been implicated in a variety of physiological and pathophysiological processes. These include mediating mitogenic responses initiated by growth factor receptors, control of cellular architecture through cytoskeletal reorganization, the UV and stress response, mitotic functions, and the induction of tumors (for review, see Ref. 1). While the biological roles of the Src family kinases are not known, it is well established that the activities of these kinases are regulated, in part, by the phosphorylation state of the negative regulatory tyrosine residue corresponding to Tyr-527 of p60\( ^{src} \). Phosphorylation of this residue by Csk or Csk-like kinases represses catalytic activity (4). Preventing phosphorylation of this residue either by association with the polyoma virus middle T-antigen or by mutation to phenylalanine or dephosphorylation of this residue by protein-tyrosine phosphatases results in increased catalytic and transforming activity (5–10). The identity of such phosphatases is by and large unknown. As mentioned above, the hematopoietic cell protein-tyrosine phosphatase (PTP)\(^1 \) CD45 regulates the phosphorylation state and activity of p56\( ^{ck} \) and p59\( ^{fyn} \) in T cells (11–15). Presumably, there are other PTPs that regulate the Src family kinases in cells lacking CD45. One possible candidate is PTP\( \alpha \), a receptor-type PTP. PTP\( \alpha \) is a widely expressed protein that differs from most other receptor-like PTPs in having a very short extracellular domain with no adhesion motifs (16–19). Overexpression of PTP\( \alpha \) leads to cell transformation and to neuronal differentiation in rat embryonic fibroblasts and in P19 carcinoma cells, respectively (20, 21). This is similar to the actions of overexpressed epidermal growth factor receptor in A431 and PC12 cells (22, 23), suggesting that PTP\( \alpha \) may normally play a role in stimulating cell proliferation. The intracellular mediators of PTP\( \alpha \) signaling are not known. The tyrosine kinase pp60\(^{c-src} \) is a candidate PTP\( \alpha \) substrate since PTP\( \alpha \) overexpression in rat embryo fibroblasts and P19 cells results in pp60\(^{c-src} \) dephosphorylation and activation (20, 21). PTP\( \alpha \) may also exert some of its cellular effects through its ability to bind the adaptor protein Grb2 (24–27). Downstream components in a PTP\( \alpha \) signaling pathway may include mitogen-activated protein kinase and the transcription factor c-Jun, both of which are activated in PTP\( \alpha \)-overexpressing rat embryo fibroblast cells (28). Whether PTP\( \alpha \) mediates the dephosphorylation of other cellular proteins besides pp60\(^{c-src} \) is unknown.

The similar structure and mode of regulation of Src family kinases suggest that other members of this family may be PTP\( \alpha \) substrates. The identification of PTP\( \alpha \) substrates is an important step in elucidating the biological role of PTP\( \alpha \). In this study, we have investigated the action of PTP\( \alpha \) toward p59\( ^{fyn} \), prompted by a combination of reasons. First, besides pp60\(^{c-src} \), only the Src family kinases p59\( ^{fyn} \) and p62\(^{ck} \) share a broad expression pattern with PTP\( \alpha \) (1). In addition, PTP\( \alpha \) is highly expressed in brain (18, 29), and PTP\( \alpha \), pp60\(^{c-src} \), and p59\( ^{fyn} \) are implicated in or associated with certain neuronal functions and neuronal differentiation (PTP\( \alpha \) (21, 30) and pp60\(^{c-src} \) (31, 32)), axonal growth (pp60\(^{c-src} \) (33) and p59\( ^{fyn} \) (34)), myelination (p59\( ^{fyn} \) (35)), and spatial learning and memory (p59\( ^{fyn} \) (36)). Second, together with pp60\(^{c-src} \), p59\( ^{fyn} \) is well defined in terms of its cellular actions. While studies in mutant mice show that Src and Fyn kinases have a high degree of functional redundancy (37), nevertheless, they also have specific and distinct functions (for example, in cytoskeletal organization (38) and adhesion molecule-directed axonal growth (33, 34)). It is thus important to define which specific intermediate signaling molecules may mediate a spectrum of PTP\( \alpha \)-directed cellular events.

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\( ^{1} \) The abbreviations used are: PTP, protein-tyrosine phosphatase; VSVG, vesicular stomatitis virus glycoprotein; Pipes, 1,4-piperazinediethanesulfonic acid; Mes, 4-morpholinethanesulfonic acid.

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Interaction of PTPα and p59<sup>fyn</sup>

**EXPERIMENTAL PROCEDURES**

**Expression Plasmids**—Numbering of the PTPα amino acid sequence is according to Krueger et al. (17). The expression vector pXJ41-PTPα-neo, encoding full-length PTPα, has been described (20). The mutagenesis altering the essential cysteine residues to serine residues in the amino acid sequences RVGHKL and MRNLKG found at either end of a 29-amino acid C-terminal fragment of VSVG (40) were used in a polymerase chain reaction with VSVG cDNA (a gift from Dr. S. H. Wong) as template. The primer sequences were 5'-GCGGTTAAATTAACTTTCCAA-GCGGTTAATTAACCGAGTT-9 and 5'-GGCGTTAATTAACTTTCCAA-GTGGTGTTCTCC-GTTCGTTTCTACT-3' (reverse). The amplified fragment was inserted into a unique PstI site in the PTPα cDNAs of pXJ41-neo, permitting the expression of PTPα proteins in a VSVG-tagged cell line. Cell extracts were prepared by lysing cells either in buffer A (50 mM NaCl, 0.2 mM Na<sub>2</sub>VO<sub>3</sub>, 1% Triton X-100, 10 μg/ml aprotinin, and 0.1 mM phenylmethylsulfonyl fluoride) (see Figs. 2 and 4C) or in modified radioimmune precipitation assay buffer (10 mM sodium phosphate (pH 7.0), 150 mM NaCl, 1 mM EDTA, 50 mM NaF, 0.1 mM Na<sub>2</sub>VO<sub>3</sub>, 1% Nonidet P-40, 0.1% SDS, 1% sodium deoxycholate, 10 μg/ml aprotinin, and 0.1 mM phenylmethylsulfonyl fluoride) (see Figs. 1 and 3) for 60 min at 4 °C. Cytosol and Triton X-100-solubilized membranes were isolated from the cytosol fraction using a 100,000 g pellet. The empty expression plasmid pXJ41-neo was used to normalize the amount of DNA in each transfection.

**Western Blots and Immunoprecipitations from Transfected Cells**—In experiments that did not involve the association of PTPα (or CD45) and p59<sup>fyn</sup>, cell extracts were prepared by lysing cells either in buffer A (50 mM Tris-Cl (pH 7.2), 150 mM NaCl, 0.2 mM Na<sub>2</sub>VO<sub>3</sub>, 1% Triton X-100, 10 μg/ml aprotinin, and 0.1 mM phenylmethylsulfonyl fluoride) (see Figs. 2 and 4C) or in modified radioimmune precipitation assay buffer (10 mM sodium phosphate (pH 7.0), 150 mM NaCl, 1 mM EDTA, 50 mM NaF, 0.1 mM Na<sub>2</sub>VO<sub>3</sub>, 1% Nonidet P-40, 0.1% SDS, 1% sodium deoxycholate, 10 μg/ml aprotinin, and 0.1 mM phenylmethylsulfonyl fluoride) (see Figs. 1 and 3) for 60 min at 4 °C. Cytosol and Triton X-100-solubilized membranes were isolated from the cytosol fraction using a 100,000 g pellet. The empty expression plasmid pXJ41-neo was used to normalize the amount of DNA in each transfection.

**Cell Culture and Transient Transfections**—Cos-1 cells were obtained from American Type Culture Collection (Rockville, MD). Cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and penicillin/streptomycin in an atmosphere of 5% CO<sub>2</sub> at 37 °C. Cos-1 cells at 50–70% confluency (100-mm dishes) were transfected with plasmid DNA by liposome-mediated transfection with 30 μl (1 μg/ml) of Lipofectamine<sup>®</sup> or LipofectAMINE<sup>®</sup> reagent (Life Technologies, Inc.) for 5–6 h as described by the manufacturer and maintained in nonselective medium until the appropriate time and were purified by high pressure liquid chromatography (Biotechnology Center, National University of Singapore). The phosphopeptide or peptide was covalently coupled to CNBr-activated Sepharose-4B (Amersham Pharmacia Inc.) and added to 300 μg of whole cell lysates (prepared with radioimmune precipitation assay buffer described above) of COS-1 cells transfected with p59<sup>fyn</sup>-expression plasmid alone or in combination with pTPα cDNA. After incubation for 2 h at 4 °C, the Sepharose beads were washed twice each with radioimmune precipitation assay buffer in the presence and absence of SDS/30% deoxycholate, respectively, and resolved by electrophoresis on a 10% SDS-polyacrylamide gel. Immunoblot analysis was as described above.

**Kinase Assays**—The kinase assays were performed in 20-μl reactions containing 10 mM Pipes (pH 7.0), 5 mM MnCl<sub>2</sub>, 0.5 mM dithiothreitol, 0.25 mM Na<sub>3</sub>VO<sub>4</sub>, and 5 μCi of [γ-<sup>32</sup>P]ATP at 37 °C for 10 min. The reactions were stopped with sample loading buffer, heated at 100 °C for 5 min, resolved by 9% SDS-polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride membranes, and autoradiographed.

**Phosphatase Assays**—Experiments in which PTP activity was measured, Na<sub>2</sub>VO<sub>3</sub> was omitted from the cell lysis buffer. PTP activity was measured at 30 °C in reactions containing 50 mM Mes (pH 6.0, 0.5 mmol/ml bovine serum albumin, 0.5 mM dithiothreitol, and 2.5–5 μM phosphorysoryl-RR-Src peptide (RRLIEDAEY(P)AARG, corresponding to the sequence encompassing Tyr-416 of p60<sup>src</sup> and phosphorylated as described (39). The specific activity of the substrate ranged between 3000 and 4000 cpm/ml of RR-Src. The reaction was carried out for 3 min unless otherwise indicated.

**RESULTS**

**PTPα Effects Dephosphorylation of p60<sup>src</sup> and p59<sup>fyn</sup>**—The ability of PTPα to dephosphorylate p60<sup>src</sup> and p59<sup>fyn</sup> was assessed in COS-1 cells cotransfected with both kinases. Anti-phosphotyrosine probing of p59<sup>fyn</sup> and p60<sup>src</sup> immunoprecipitates demonstrated that PTPα expression resulted in tyrosine dephosphorylation of both enzymes (Fig. 1, lanes A and B). Blotting of these cell lysates with anti-cst.1, an antibody that recognizes both p59<sup>fyn</sup> and p60<sup>src</sup> equally well (42), showed that similar amounts of these kinases were expressed with PTPα (data not shown). As p59<sup>fyn</sup> represents a novel potential substrate for PTPα, we characterized the catalytic action of PTPα toward p59<sup>fyn</sup> in more detail. The extent of p59<sup>fyn</sup> dephosphorylation increased as increasing amounts of PTPα cDNA were transfected, reaching a plateau of 80–90% tyrosine dephosphorylation at a ratio of 1:8 between PTPα and p59<sup>fyn</sup>.
dephosphorylation (Fig. 2A). Dephosphorylation was completely dependent on the catalytic activity of PTPα since a PTPα mutant, PTPα(C414S/C704S), in which the essential cysteine residues in the active sites of both catalytic domains of PTPα were mutated to serine residues, was unable to effect p59fyn dephosphorylation (Fig. 2A).

**Coexpression of PTPα and p59fyn Results in p59fyn Activation—** Besides tyrosine dephosphorylation of p59fyn, the coexpression of PTPα resulted in kinase activation of p59fyn. As shown in Fig. 2B, when p59fyn immunoprecipitates from cells coexpressing PTPα were used in an immunocomplex kinase assay, increasing p59fyn autophosphorylation was observed with increasing expression of PTPα. A maximum 5-fold increase in kinase activity was obtained (Fig. 2B, lanes 5 and 6) at the same PTPα/p59fyn cDNA ratio observed to give maximal p59fyn dephosphorylation (Fig. 2A). No increase in the kinase activity of p59fyn was measured when catalytically inactive PTPα was expressed with p59fyn (data not shown).

**p59fyn Dephosphorylation and Activation Are Accompanied by Increased SH2 Domain Accessibility—** Dephosphorylation of the C-terminal tyrosine residue of Src family kinases is linked to kinase activation (5–9) and correlates with increased c-Src SH2 domain and Lck SH2 domain accessibility during mitosis and T cell activation, respectively (43, 44). These observations support a model of kinase activation where disruption of an intramolecular association between the C-terminal phosphotyrosyl peptide and the SH2 domain of the kinase results in catalytic activation as well as novel interactions of the SH2 domain with other phosphotyrosyl proteins (2, 3). The effect of PTPα on p59fyn SH2 domain accessibility was examined by determining the ability of p59fyn to bind to a synthetic phosphopeptide representing the C-terminal Tyr-527 peptide of pp60src. This peptide is identical to the C-terminal sequence surrounding Tyr-531 of p59fyn except for the replacement of alanine in position 2 with serine (45). As shown in Fig. 3, PTPα-induced dephosphorylation of p59fyn (Fig. 3C) correlated with a 3-fold increase in the amount of Fyn protein precipitated from cell lysates with the Src Tyr-527 phosphopeptide coupled to Sepharose beads (Fig. 3D, bottom panel). A control precipitation using an unphosphorylated Src Tyr-527 peptide-Sepharose conjugate contained barely detectable but equivalent amounts of Fyn protein from p59fyn- and PTPα/p59fyn-expressing cells (Fig. 3D, top panel). While the site(s) of p59fyn dephosphorylation remains to be mapped, the increased p59fyn catalytic activity and SH2 availability for binding are consistent with a PTPα-mediated dephosphorylation of the C-terminal Tyr-531 of p59fyn.

**Specificity of PTPα in Effecting p59fyn Dephosphorylation and Activation—** To examine whether the PTPα-mediated p59fyn dephosphorylation was a specific effect of PTPα or merely reflected a nonspecific increase in PTP activity at the cell membrane, the tyrosine phosphorylation state of p59fyn was analyzed upon coexpression with another receptor-like
PTP, CD45. CD45 is a hematopoietic cell-specific molecule required for T and B cell activation (46–48) and can dephosphorylate the Src family kinases p59<sup>fn</sup> and p56<sup+lck</sup> in T cells (11–15) and p62<sup>shc</sup> in B cells (49). Fractionation of transfected COS cells into solubilized membrane and cytosol followed by Western blotting showed that, like PTP<sub>a</sub>, CD45 was localized to membranes (bottom panel). As expected, a majority of the Fyn protein was also associated with membranes (Fig. 4A, top panel). Elevated membrane PTP activity was measured in PTP<sub>a</sub>- or CD45-expressing cells (Fig. 4B), demonstrating that both receptor PTPs are enzymatically active and that comparable levels of phosphatase activity are seen in both cell types. However, immunoprecipitated p59<sup>fn</sup> was tyrosine-dephosphorylated in PTP<sub>a</sub>-expressing cells (Fig. 4C, compare lanes 1 and 2), whereas no reduction in the tyrosine phosphate content of p59<sup>fn</sup> from CD45-expressing cells was detected (compare lanes 1 and 3). Even when the amount of CD45 cDNA was increased 4-fold over that used for the experiments shown in Fig. 4, no dephosphorylation of coexpressed p59<sup>fn</sup> was observed (data not shown). This is similar to another report that CD45 cannot effect p56<sup+lck</sup> dephosphorylation when expressed in non-lymphoid cells (15). The p59<sup>fn</sup> dephosphorylation observed in the presence of PTP<sub>a</sub>, but not CD45, correlated with elevated kinase activity of Fyn in PTP<sub>a</sub>-expressing cells (as described above) and no alteration in the kinase activity of p59<sup>fn</sup> from CD45-expressing cells (data not shown). Thus, increased membrane PTP activity is not in itself sufficient to effect p59<sup>fn</sup> dephosphorylation and activation, indicating that the PTP<sub>a</sub>-mediated dephosphorylation and activation of p59<sup>fn</sup> reflect a specific effect of PTP<sub>a</sub>.

**Association of PTP<sub>a</sub> and p59<sup>fn</sup>—**To further investigate the interaction of PTP<sub>a</sub> and p59<sup>fn</sup>, we examined whether these two proteins underwent any form of association. To enable immunoprecipitation of PTP<sub>a</sub>, a 29-amino acid epitope from VSVG was inserted into the PTP<sub>a</sub> extracellular region to create VSVG-PTP<sub>a</sub>. Cells expressing p59<sup>fn</sup> alone or in conjunction with VSVG-PTP<sub>a</sub> were lysed under mild detergent conditions, and PTP<sub>a</sub> was immunoprecipitated with anti-VSVG antibodies. Probing of the immunoprecipitates with anti-VSVG antibodies detected two forms of VSVG-PTP<sub>a</sub>: a broad diffuse band migrating at ~130 kDa, consistent with the size of wild-type glycosylated PTP<sub>a</sub>, and a sharper band at ~100 kDa, consistent with the size of incompletely glycosylated PTP<sub>a</sub> (50) (Fig. 5A, top panel, lanes 7 and 8). Probing of these samples and whole cell lysates with anti-p59<sup>fn</sup> antibodies revealed equal amounts of Fyn protein in cell lysates expressing p59<sup>fn</sup> alone or together with VSVG-PTP<sub>a</sub> (Fig. 5A, bottom panel, lanes 2 and 4) and a significant amount of p59<sup>fn</sup> in the anti-VSVG immunoprecipitate from the PTP<sub>a</sub>/p59<sup>fn</sup>-coexpressing cells (lane 8). The presence of this p59<sup>fn</sup> in the VSVG immunoprecipitate was due to its interaction with VSVG-PTP<sub>a</sub> since only a weak p59<sup>fn</sup> signal (likely due to nonspecific sticking) was detected in anti-VSVG immunoprecipitates prepared from cells expressing p59<sup>fn</sup> in the absence of VSVG-PTP<sub>a</sub> (Fig. 5A, bottom panel, lane 6). In addition, in vitro assay of kinase activity in the anti-VSVG immunoprecipitates detected enhanced phosphorylation of a protein (Fig. 5B, lane 5) that comigrated with autophosphorylated p59<sup>fn</sup> produced in vitro kinase assay of an anti-Fyn immunoprecipitate from p59<sup>fn</sup>-expressing cells (lane 1). These and subsequent experiments to examine the association of PTP<sub>a</sub> and p59<sup>fn</sup> were
Association of PTPα and a Catalytic Mutant of PTPα—

The mechanism and temporal occurrence of PTPα and p59\(^{\text{fn}}\) association (pre- or post-dephosphorylation) were examined using a catalytically inactive form of PTPα. Mutation of the essential cysteine residue in the conserved catalytic domain of PTPs creates an enzymatically inactive PTP (51, 52), which has been shown to bind to and “trap” phosphotyrosyl substrates (53–55). A VSVG-PTPα double mutant in which the essential cysteine residues in both the membrane proximal and distal catalytic domains were mutated to serine residues (C414S/C704S) was produced. If association involves the recognition of phosphotyrosine sites in p59\(^{\text{fn}}\) as a pre-dephosphorylation event, then such interaction might be stabilized and enhanced with enzymatically inactive PTPα. However, if p59\(^{\text{fn}}\) dephosphorylation is prerequisite for PTPα-p59\(^{\text{fn}}\) association, then the phosphatase-kinase complex will not be formed with the enzymatically inactive mutant. No dephosphorylation of p59\(^{\text{fn}}\) was detected upon coexpression with mutant VSVG-PTPα(C414S/C704S) (data not shown, but see Fig. 2A). Nevertheless, p59\(^{\text{fn}}\) was detected in immunoprecipitates of VSVG-PTPα(C414S/C704S) at a level equivalent to that of p59\(^{\text{fn}}\) in immunoprecipitates of catalytically active VSVG-PTPα (Fig. 7). Thus, a conventional substrate-trapping technique fails to enhance PTPα-p59\(^{\text{fn}}\) interaction, suggesting that additional or alternative regions of these proteins are responsible for association. Also, the finding that p59\(^{\text{fn}}\) and PTPα can associate independently of PTPα activity, and thus in the absence of or prior to dephosphorylation, indicates that PTPα is suitably positioned to directly utilize p59\(^{\text{fn}}\) as a substrate.

Association of Endogenous PTPα with p59\(^{\text{fn}}\)—

The above studies were carried out with COS cells ectopically expressing PTPα and p59\(^{\text{fn}}\). To determine whether association occurred between these proteins when present at endogenous levels, we examined whether they could be co-immunoprecipitated from mouse brain lysates. Brain was chosen as the tissue source because PTPα is highly expressed in brain (18, 29) and because neuronal functions of both PTPα (21, 30) and p59\(^{\text{fn}}\) (34–36) have been reported. None of the PTPα antibodies available to us immunoprecipitated PTPα very efficiently, based on comparisons with the level of PTPα detected by Western blotting of brain lysates (data not shown). Some PTPα could be precipitated (Fig. 8, lane 2) with a polyclonal antibody (3860) raised against a synthetic peptide corresponding to the C-terminal 18 amino acids of PTPα, and specific immunoprecipitation of PTPα was blocked by preincubation of the antibody with recombinant purified protein (PTPα-D2) comprising the membrane distal catalytic domain and C terminus of PTPα. PTPα was detected in p59\(^{\text{fn}}\) immunoprecipitates (Fig. 8, lane 3), but not in immunoprecipitates of Csk (lane 4), a non-receptor tyrosine kinase structurally similar to p59\(^{\text{fn}}\). The above immunoprecipitations were probed with anti-PTPα antibody (3897) raised against recombinant PTPα-D2. Stripping...
Interaction of PTPα and p59<sup>fyn</sup>

Fig. 6. PTPα (but not CD45) associates with p59<sup>fyn</sup>. Cells were transfected with 2 μg of p59<sup>fyn</sup> cDNA and 2 μg of PTPα cDNA or 4 μg of CD45 cDNA, with the total amount of DNA in each transfection equalized by the addition of empty plasmid. A, whole cell lysates (lanes 1–3) and immunoprecipitates (IP) of p59<sup>fyn</sup> from 800 μg of lysates of COS-1 cells expressing p59<sup>fyn</sup>, VSVG-PTPα, or p59<sup>fyn</sup> and VSVG-PTPα (lanes 4–6) were probed with anti-VSVG (top panel) and anti-p59<sup>fyn</sup> (bottom panel) antibodies. B, whole cell lysates (lanes 1–3) and immunoprecipitates of p59<sup>fyn</sup> from 800 μg of lysates of COS-1 cells expressing p59<sup>fyn</sup>, CD45, or p59<sup>fyn</sup> and CD45 (lanes 4–6) were probed with anti-CD45 (top panel) and anti-p59<sup>fyn</sup> (bottom panel) antibodies. C, phosphatase assays of phosphotyrosyl-RR-Src peptide were performed with p59<sup>fyn</sup> immunoprecipitates from lysates of COS-1 cells expressing p59<sup>fyn</sup> (a, open diamonds), VSVG-PTPα (b, open squares), p59<sup>fyn</sup> and VSVG-PTPα (c, closed squares), CD45 (d, open triangles), or p59<sup>fyn</sup> and CD45 (e, open circles). The inset shows the RR-Src phosphatase activity of these cell lysates (10<sup>4</sup> cpm released per 3 min/μg of lysate) prior to immunoprecipitation.

and reprobing of lanes 1–4 with the anti-PTPα C-terminal antibody (3680, used for PTPα immunoprecipitations; see above) gave the same results (data not shown). This association of PTPα with p59<sup>fyn</sup> was observed in repeated experiments and demonstrates that these proteins are physiologically associated.

**DISCUSSION**

We have confirmed that PTPα dephosphorylates pp60<sup>src</sup> in vivo and found that PTPα can also dephosphorylate the related Src family kinase p59<sup>fyn</sup>. The concomitant increase in p59<sup>fyn</sup> kinase activity and increase in accessibility of the p59<sup>fyn</sup> SH2 domain are consistent with a PTPα-mediated dephosphorylation of the C-terminal tyrosine residue thought to be critical in regulating the activity of p59<sup>fyn</sup>. In contrast, the expression of the receptor-like PTP CD45 in this system did not result in p59<sup>fyn</sup> dephosphorylation or activation, demonstrating a specific action of PTPα on p59<sup>fyn</sup>. Previously, PTPα activity has mainly been assayed for artificial substrates in vitro using immunoprecipitated PTPα or bacterially expressed forms of cytosolic PTPα (24, 39, 56, 57). This characterization of a cellular assay of PTPα activity will be useful in the future assessment of the effects of various structural mutants of PTPα on its enzymatic function.

An important question regarding the activation of Src family kinases by PTPα is whether these kinases are directly dephosphorylated by PTPα and thus represent PTPα substrates. Potential PTPα substrates might be complexed with the phosphatase, as is the case with many tyrosine kinases and their substrates, or with CD45 and its substrates in T (58–62) and B (63) cells. Co-immunoprecipitation experiments with transfected cells demonstrated that PTPα and p59<sup>fyn</sup> were consistently found to be associated with one another. This was not merely an artifact of heterologous expression of these proteins since CD45 and p59<sup>fyn</sup> were not found in association under the same experimental conditions. Likewise, what appears to be an incompletely glycosylated (and thus perhaps inappropriately localized) form of PTPα did not associate with p59<sup>fyn</sup>. In addition, PTPα was detected in p59<sup>fyn</sup> immunoprecipitates from mouse brain. Although PTPα-p59<sup>fyn</sup> association is suggestive of a direct enzyme-substrate relationship, at present it is unclear whether these physical and functional actions of PTPα are linked. If so, two possibilities are that association enables the subsequent dephosphorylation and activation of p59<sup>fyn</sup> or that activated p59<sup>fyn</sup> directly or indirectly modifies PTPα to promote PTPα-p59<sup>fyn</sup> association, perhaps after tyrosine phosphorylation of the phosphatase creates p59<sup>fyn</sup>-binding sites. Our evidence supports the former scenario since the association of a
catalytically inactive form of PTPα with p59<sup>fyn</sup> indicates that this physical interaction does not require prior dephosphorylation/activation of p59<sup>fyn</sup>. We are generating truncated forms of PTPα to define the region(s) involved in p59<sup>fyn</sup> binding. Regions of p59<sup>fyn</sup> that associate with a variety of other signaling molecules include the SH3 and SH2 domains (for examples, see Refs. 64–67) and the unique N-terminal region (68, 69). A proline-rich sequence similar to the consensus sequence for SH3 binding (70–72) is found in PTPα (RKYPPLP, amino acids 188–194). As PTPα is tyrosine-phosphorylated in the cell (25, 26), this could provide sites for SH2 binding. Alternatively, PTPα-p59<sup>fyn</sup> association may occur through other regions or intermediary proteins.

Besides the association with p59<sup>fyn</sup> described here, PTPα can associate with the adaptor molecule Grb2 (24–27). The latter complex is formed upon phosphorylation of a tyrosine residue in the tail region of PTPα and binding by the SH2 domain of Grb2. A direct or indirect association also occurs between the C-terminal SH3 domain of Grb2 and a non-proline-rich region near the active site of the membrane proximal catalytic domain of PTPα, but is not observed in the absence of the PTPα-Grb2(SH3) interaction. One effect of PTPα-Grb2(SH3) binding is postulated to be the inhibition of the catalytic activity of PTPα through the obstruction of substrate binding (26). Tyrosine phosphorylation of PTPα can be catalyzed by pp60<sup>src</sup> (24), and it will be of interest to see if p59<sup>fyn</sup> can phosphorylate PTPα at the same site to result in Grb2 binding. We have observed enhanced tyrosine phosphorylation of PTPα upon coexpression with p59<sup>fyn</sup><sup>2</sup> although the site(s) of phosphorylation is unknown. If this phosphorylation occurs at the appropriate C-terminal site, PTPα-catalyzed activation of pp60<sup>src</sup> and/or p59<sup>fyn</sup> would activate kinase-mediated downstream signaling events while also permitting the feedback inhibition of PTPs by effecting Grb2 binding. Regardless of whether p59<sup>fyn</sup> activation and Grb2 binding are linked, it will be of interest to see whether p59<sup>fyn</sup> and Grb2 binding to PTPα can occur on the same PTPα molecule or are mutually exclusive.

Previous studies have suggested that the cell transformation and retinoic acid-induced neuronal differentiation observed in certain cell types upon PTPα expression may be mediated through pp60<sup>src</sup> (20, 21). Here we have provided further evidence that PTPα is a physiological regulator of the Src family kinases and, in particular, that p59<sup>fyn</sup> is an in vivo substrate of PTPα. As with pp60<sup>src</sup>, prevention of C-terminal Tyr-531 phosphorylation of p59<sup>fyn</sup> (by mutation of this tyrosine to phenylalanine) results in an oncoprotein, which upon overexpression transforms rodent fibroblasts (73). The altered phenotype of PTPα-expressing cells may be due to the increased kinase activity of p59<sup>fyn</sup>. Alternatively, PTPα-induced transformation and differentiation may be a consequence of the combined or synergistic effect of increased p59<sup>fyn</sup> and pp60<sup>src</sup> catalytic activity. The association of PTPα with p59<sup>fyn</sup> in brain, in conjunction with the presence of both proteins in neuronal cells such as cerebellar granule cells (29, 74, 75) and dorsal root ganglia (34, 76), suggests that p59<sup>fyn</sup> could be a component of as yet unknown PTPα signaling pathways of neuronal development.

Dephosphorylation and activation of Src family kinases have been demonstrated or implicated in studies of the signaling pathways of two receptor-like PTPs, PTPα and CD45. It is conceivable that these and other non-adhesion receptor-type PTPs share a common mode of signaling, with specificity determined by the respective spatial and temporal patterns of gene expression of the receptor-type PTPs and Src family kinases.

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