Platelet-derived Growth Factor (PDGF) Stimulates the Association of SH2-Bβ with PDGF Receptor and Phosphorylation of SH2-Bβ*

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We recently identified SH2-Bβ as a JAK2-binding protein and substrate involved in the signaling of receptors for growth hormone and interferon-γ. In this work, we report that SH2-Bβ also functions as a signaling molecule for platelet-derived growth factor (PDGF). SH2-Bβ fused to glutathione S-transferase (GST) bound PDGF receptor (PDGFR) from PDGF-treated but not control cells. GST fusion protein containing only the SH2 domain of SH2-Bβ also bound PDGFR from PDGF-treated cells. An Arg to Glu mutation within the FVLRQS motif in the SH2 domain of SH2-Bβ inhibited GST-SH2-Bβ binding to tyrosyl-phosphorylated PDGFR. The N-terminal truncated SH2-Bβ containing the entire SH2 domain interacted directly with tyrosyl-phosphorylated PDGFR from PDGF-treated cells but not unphosphorylated PDGFR from control cells in a Far Western assay. These results suggest that the SH2 domain of SH2-Bβ is necessary and sufficient to mediate the interaction between SH2-Bβ and PDGFR. PDGF stimulated coimmunoprecipitation of endogenous SH2-Bβ with endogenous PDGFR in both 3T3-F442A and NIH3T3 cells. PDGF stimulated the rapid and transient phosphorylation of SH2-Bβ on tyrosines and most likely on serines and/or threonines. Similarly, epidermal growth factor stimulated the phosphorylation of SH2-Bβ; however, phosphorylation appears to be predominantly on serines and/or threonines. In response to PDGF, SH2-Bβ associated with multiple tyrosyl-phosphorylated proteins, at least one of which (designated p84) does not bind to PDGFR. Taken together, these data strongly argue that, in response to PDGF, SH2-Bβ directly interacts with PDGFR and is phosphorylated on tyrosine and most likely on serines and/or threonines, and acts as a signaling protein for PDGF.

We recently identified the SH2β domain-containing molecule, SH2-Bβ, as a substrate of JAK2 involved in signaling by growth hormone (GH) and interferon-γ (1). Receptors for GH and interferon-γ are members of the cytokine receptor family and are known to bind JAK tyrosine kinases (JAK2 for GH and both JAK1 and JAK2 for interferon-γ). After GH binding, JAK2 is activated and tyrosyl-phosphorylates its associated GH receptor as well as JAK2 itself (2, 3). As a consequence of JAK2 autophosphorylation, SH2-Bβ is recruited into receptor/JAK2 complexes at least in part via the direct interaction of the SH2 domain of SH2-Bβ with phosphotyrosine containing motifs in JAK2 (4). GH promotes not only the association of SH2-Bβ with tyrosyl-phosphorylated JAK2, but also the tyrosyl phosphorylation of SH2-Bβ (1). SH2-Bβ also appears to be phosphorylated on serine(s) and/or threonine(s), even in the absence of ligand stimulation (1). These findings suggested that SH2-Bβ, which contains multiple potential sites for protein-protein interaction in addition to its SH2 domain (9 tyrosines, a pleckstrin homology (PH) domain, and multiple proline-rich motifs (Fig. 1A), serves as an adapter protein and recruits additional signaling molecules into cytokine receptor-JAK2 complexes (1). Many signaling molecules are shared by cytokine receptors and receptor tyrosine kinases, particularly those signaling molecules containing SH2 domains. For example, Shc, Grb2, and phosphatidylinositol 3′-kinase are important signaling molecules in the biological actions of GH (2, 4) and platelet-derived growth factor (PDGF) (5, 6). In support of SH2-Bβ serving as a signaling molecule for receptor tyrosine kinases, SH2-Bβ was found to interact with receptors for insulin and insulin-like growth factor-1 (1, 7, 8). In this work, we demonstrate that PDGF stimulates association of SH2-Bβ with PDGFR receptor (PDGFR), and phosphorylation of SH2-Bβ. We also show that SH2-Bβ associates, not via the PDGFR, with a tyrosyl-phosphorylated, 84-kDa protein. These results provide strong evidence that SH2-Bβ is a previously unknown signaling molecule for PDGF.

EXPERIMENTAL PROCEDURES

Cells and Reagents—The stock of 3T3-F442A fibroblasts was kindly provided by H. Green (Harvard University, Cambridge, MA). Recombinant human GH was a gift of Eli Lilly and Co. Recombinant human PDGF-BB was from Life Technologies, Inc. Recombinant human PDGF-AA was from Collaborative Biomedical Products. Glutathione-agarose beads were from Sigma. Recombinant protein A-agarose was from Repligen. Alkaline phosphatase, aprotinin, leupeptin, and Triton X-100 were from Boehringer Mannheim. Protein phosphatase 2A (PP2A) was from Upstate Biotechnology, Inc. Enhanced chemiluminescence (ECL) detection system was from Amersham Corp. Antibodies to rat SH2-Bβ (αSH2-B) were raised against GST-SH2-Bβc as described previously (1), and used at a dilution of 1:100 for immunoprecipitation and 1:15,000 for immunoblotting. Anti-JAK2 antiserum (αJAK2) was raised in rabbits against a synthetic peptide corresponding to amino acids 758–776 of murine JAK2 (9) and was used at a dilution of 1:500 for immunoprecipitation and 1:15,000 for immunoblotting. Monoclonal anti-phosphotyrosine antibody 4G10 (αPY) and polyclonal antibody against human PDGF receptor (αPDGFR, recognizing both α and β subunits) were from Upstate Biotechnology, Inc. and used at a dilution of 1:7,500 and 1:1,000 for immunoblotting, respectively. αPDGFR was used at a dilution of 1:100 for immunoprecipitation.

Methods—3T3-F442A fibroblasts were treated for 10 min with 25 ng/ml PDGF-BB, vehicle, or other ligands as indicated. For GST fusion protein pull-down assays, whole cell lysates were precipitated with GST
fused proteins immobilized on glutathione-agarose beads and subsequently immunoblotted with αPDGFR or αPY as described previously (1). GST fusion proteins containing SH2-Bβ or mutant SH2-Bβ were prepared as described previously (1). Arg within the FLVQRG motif in the SH2 domain of SH2-Bβ was mutated to Glu using a site-directed mutagenesis kit (Stratagene), and the mutation was confirmed by DNA sequencing. The mutant SH2-Bβ (SH2-Bβ-R-E) was subcloned into pGEX-KG to generate a GST fusion protein. For immunoprecipitations, cell lysates were incubated with the indicated antibody on ice for 2 h. The immune complexes were collected on protein A-agarose (50 μl) during a 1-h incubation at 4 °C. In some experiments, αSH2-B immunoprecipitates were dephosphorylated by alkaline phosphatase or PP2A as described previously (1). The immobilized proteins were immunoblotted with the indicated antibody. Some membranes were stripped by incubation at 55 °C for 30–60 min in stripping buffer (100 mM Tris-HCl, pH 6.7) and reprobed with a different antibody. For Far Western blotting, PDGFR was immunoprecipitated with αPDGFR from solubilized 3T3-F442A fibroblasts, subjected to SDS-PAGE, and transferred onto nitrocellulose. The nitrocellulose was incubated with GST-SH2-Bβc (1.5 μg/ml) at 4 °C overnight. After extensive washing, the membrane was immunoblotted with αSH2-B. The blot was stripped and reprobed with αPDGFR and αPY sequentially.

RESULTS

SH2-Bβ Binds to PDGFR Only from PDGFR-treated Cells—To determine whether SH2-Bβ serves as a signaling molecule for PDGFR, we first tested whether SH2-Bβ binds to PDGFR. 3T3-F442A fibroblasts, which express endogenous PDGFR (10), were deprived of serum overnight and treated with 25 ng/ml PDGF-BB or vehicle for 10 min. Cell lysates were incubated with immobilized GST or GST fusion protein containing full-length SH2-Bβ, N-terminally truncated SH2-Bβ (SH2-Bβc), or the SH2 domain of SH2-Bβ (Fig. 1A), and immunoblotted with αPDGFR. GST-SH2-Bβ bound to PDGFR in a ligand-dependent manner (Fig. 1B, lanes 1 and 2). GST-SH2-Bβc (Fig. 1B, lanes 3 and 4) and GST-SH2 domain of SH2-Bβ (Fig. 1B, lanes 5 and 6) also bound PDGFR from PDGFR- but not vehicle-treated cells. Reprobing with αPY showed that PDGFR that is associated with SH2-Bβ or truncated SH2-Bβ is tyrosyl-phosphorylated (data not shown). In contrast, GST alone did not bind PDGFR (Fig. 1B, lane 7). These results suggest that SH2-Bβ interacts with activated, tyrosyl-phosphorylated PDGFR, and that the SH2 domain of SH2-Bβ may mediate the interaction between SH2-Bβ and PDGFR.

SH2-Bβ Binds Directly to Tyrosyl-phosphorylated PDGFR through Its SH2 Domain—To investigate whether SH2-Bβ binds PDGFR directly or indirectly through some intermediate molecule, the ability of GST-SH2-Bβc to bind PDGFR was determined by Far Western blotting. 3T3-F442A cells were treated with 25 ng/ml PDGF-BB or vehicle. PDGFR was immunoprecipitated with αPDGFR, resolved by SDS-PAGE, and transferred to nitrocellulose. The nitrocellulose was incubated first with GST-SH2-Bβc, and then with αSH2-B. GST-SH2-Bβc bound directly to PDGFR from PDGFR-treated (Fig. 2A, lane 2) but not vehicle-treated cells (Fig. 2A, lane 1), although an equal amount of PDGFR was present (Fig. 2A, lanes 3 and 4). Reprobing the same blot with αPY confirmed the PDGF-dependent tyrosyl phosphorylation of PDGFR (Fig. 2A, lanes 5 and 6). These results indicate that SH2-Bβc binds directly to tyrosyl-phosphorylated, activated PDGFR.

FIG. 1. GST-SH2-Bβ binds to PDGFR from PDGFR-treated cells. A, schematic representation of full-length and truncated SH2-Bβ. Tyrosines and the SH2 and PH domains are designated. The C-terminal gray region designates the amino acids that are not shared with SH2-Bα. B, 3T3-F442A cells were treated for 10 min with vehicle (lanes 1, 3, and 5) or 25 ng/ml PDGF-BB (lanes 2, 4, 6, and 7). Whole cell lysates were incubated with the indicated GST fusion protein immobilized on glutathione-agarose beads. Bound proteins were separated by SDS-PAGE and immunoblotted with αPDGFR. The amount of GST-SH2-Bβ was less than one-third the amount of the other GST fusion proteins.

FIG. 2. SH2-Bβ binds directly to tyrosyl-phosphorylated PDGFR via the SH2 domain of SH2-Bβ. A, 3T3-F442A cells were treated for 10 min with 25 ng/ml PDGF-BB (lanes 2, 4, and 6) or vehicle (lanes 1, 3, and 5). Solubilized proteins were immunoprecipitated with αPDGFR. The immunoprecipitated proteins were separated by SDS-PAGE and transferred onto nitrocellulose. The nitrocellulose was incubated with GST-SH2-Bβc at 4 °C overnight. After extensive washing, the nitrocellulose was immunoblotted with αSH2-B (lanes 1 and 2). The same blot was sequentially reprobed with αPDGFR (lanes 3 and 4) and αPY (lanes 5 and 6). B, 3T3-F442A (lanes 1–3) and NIH3T3 (lanes 4–6) cells were treated for 10 min with vehicle (lanes 1 and 4) or 25 ng/ml PDGF-BB (lanes 2, 3, 5, and 6). Whole cell lysates were incubated with the indicated GST fusion protein immobilized on glutathione-agarose beads. Bound proteins were separated by SDS-PAGE and immunoblotted with αPY. The migration of molecular weight standards (×10−3) (panel A) and PDGFR (panels A and B) is indicated.
To demonstrate the crucial role of the SH2 domain of SH2-Bβ in mediating the interaction of SH2-Bβ with PDGFR, we examined the ability of PDGFR to bind to GST fusion protein containing a mutant SH2-Bβ in which Glu replaced the Arg (amino acid 555) within the FLVRQS motif in the SH2 domain. This positively charged Arg within the highly conserved FLVRQS motif in SH2 domains has been shown to interact with the negatively charged phosphate on the phosphotyrosine of its binding partner (11–13). Mutation of this critical Arg has been shown to abolish the binding ability of the SH2 domain to its corresponding phosphorylation motif for several molecules (14, 15). GST fusion protein containing this mutant SH2-Bβ containing a mutant SH2-Bβ (GST-SH2-Bβ[R-E]) immobilized on glutathione-agarose beads was incubated with cell lysates of either 3T3-F442A or NIH3T3 cells. The bound proteins were separated by SDS-PAGE and immunoblotted with an αPY. GST-SH2-Bβ bound to tyrosyl-phosphorylated PDGFR from PDGF-treated NIH3T3 cells (Fig. 2, lane 5) as well as 3T3-F442A cells (Fig. 2, lane 2), indicating that the interaction of SH2-Bβ with PDGFR is not cell-type-specific. Mutation of Arg to Glu within the SH2 domain of SH2-Bβ inhibited binding of SH2-Bβ to tyrosyl-phosphorylated PDGFR from either 3T3-F442A (Fig. 2B, lane 3) or NIH3T3 cells (Fig. 2B, lane 6) cells. These results suggest that the SH2 domain of SH2-Bβ mediates the interaction between SH2-Bβ and PDGFR.

**PDGF-BB Stimulates the Association of Endogenous SH2-Bβ with PDGFR**—To examine whether endogenous SH2-Bβ associates with endogenous PDGFR in mammalian cells, 3T3-F442A fibroblasts, shown previously to express endogenous SH2-Bβ (1), were treated with PDGF-BB or vehicle. Solubilized proteins were immunoprecipitated with αSH2-B and immunoblotted with an αPDGFR. SH2-Bβ was observed to coimmunoprecipitate with PDGFR in PDGF-stimulated (Fig. 3, lane 2) but not control cells (Fig. 3, lane 1), consistent with the findings (shown in Figs. 1B and 2) that SH2-Bβ binds only to tyrosyl-phosphorylated, activated PDGFR. Pre-immune serum was unable to precipitate PDGFR from PDGF-treated cells (Fig. 3, lanes 3 and 6). Reprobing the same blot with an αPY confirmed that PDGFR associated with SH2-Bβ is tyrosyl-phosphorylated (Fig. 3, lane 5). Similarly, SH2-Bβ was detected in αPDGFR immunoprecipitates only when cells were stimulated with PDGF (data not shown). SH2-Bβ also coimmunoprecipitated with PDGFR in NIH3T3 cells in response to PDGF-BB (Fig. 3, lanes 7 and 8), further demonstrating that the association of SH2-Bβ with PDGFR is not cell-type-specific. Taken together, the results of Figs. 1–3 suggest that PDGF stimulates the recruitment of SH2-Bβ to PDGFR presumably via a direct interaction of the SH2 domain of SH2-Bβ with phosphotyrosine-containing motif(s) in the activated PDGFR.

**PDGF-BB Promotes Tyrosyl Phosphorylation of SH2-Bβ**—SH2-Bβ was previously shown to be tyrosyl-phosphorylated in response to GH and interferon-γ (1). To test whether PDGF is able to stimulate tyrosyl phosphorylation of SH2-Bβ, 3T3-F442A cells were treated with PDGF-BB, and cell lysates were immunoprecipitated with αSH2-B and immunoblotted with an αPY. PDGF-BB stimulated tyrosyl phosphorylation of SH2-Bβ (Fig. 4, upper panel; Fig. 5B, lane 4; Fig. 6, lane 4; Fig. 7, lane 3). Based upon the migration of proteins immunoblotted with an αSH2-B (Fig. 4, lower panel; Fig. 5B, lane 9; Fig. 7, lane 8), SH2-Bβ migrates as a diffuse band indicated by the bracket. For reasons discussed below, the tight band, designated p84 in Figs. 4, 5B, 6, and 7, is believed to be a tyrosyl-phosphorylated protein that coimmunoprecipitates with SH2-Bβ, and not a form of SH2-Bβ. As predicted from Figs. 1–3, tyrosyl-phosphorylated PDGFR coimmunoprecipitated with SH2-Bβ from PDGF-BB-treated cells (Fig. 4, lanes 2–7, upper panel; Fig. 5B, lanes 4 and 5; Fig. 6, lane 4; Fig. 7, lanes 2 and 3) but not from control (Fig. 4, lane 1; Fig. 5B, lanes 1–3; Fig. 6, lane 3; Fig. 7, lane 1) or GH- or EGF-treated cells (Fig. 7, lanes 4 and 5). When the blots were reprobed with an αSH2-B, PDGFB was observed to cause a significant upward shift in the mobility of SH2-Bβ (Fig. 4, lower panel; Fig. 5A, lane 2; Fig. 5B, lane 9; Fig. 7, lane 8), consistent with SH2-Bβ being phosphorylated in response to PDGF. The PDGF-BB-induced tyrosyl phosphorylation and shift in mobility of SH2-Bβ were rapid (within 1 min), transient (Fig. 4), and dose-dependent (data not shown), indicating that phosphorylation of SH2-Bβ is a tightly regulated process. Interestingly, the greatest shift in SH2-Bβ mobility was observed after 5 min of 25 ng/ml PDGF (Fig. 4, lower panel), while the tyrosyl phosphorylation of SH2-Bβ was not maximal until 15 min (Fig. 4, upper panel). Similarly, the mobility shift of SH2-Bβ was the greatest at a dose of 5 ng/ml for 15 min, but the tyrosyl phosphorylation was not maximal until 25 ng/ml (data not shown). Because the multiple SH2-Bβ bands in control and GH-treated 3T3-F442A cells have been shown to be differentially phosphorylated forms of SH2-Bβ, this discrepancy between mobility shift and αPY signal suggests that in addition to stimulating tyrosyl phosphorylation, PDGF-BB also promotes the phosphorylation of SH2-Bβ on serine(s) and/or threonine(s). Curiously, the degree of tyrosyl phosphorylation of SH2-Bβ measured using αPY Western blot-
PDGF stimulates phosphorylation of SH2-Bβ at multiple sites. A, 3T3-F442A cells were treated for 10 min with 25 ng/ml PDGF-BB (lanes 2–4) or with vehicle (lane 1), and solubilized proteins were immunoprecipitated with αSH2-B. The immunoprecipitates were incubated at 37°C for 60 min in dephosphorylation buffer with (lanes 3 and 4) or without (lanes 1 and 2) alkaline phosphatase (AP), and with (lane 4) or without (lanes 1–3) Na3VO4. The reaction mixtures were subjected to SDS-PAGE and immunoblotted with αSH2-B. B, 3T3-F442A cells were treated for 10 min with 25 ng/ml PDGF-BB or vehicle as indicated, and solubilized proteins were immunoprecipitated with αSH2-B. αSH2-B immunoprecipitates were incubated with (lanes 2, 3, 5, 7, 8, and 10–12) or without (lanes 1, 4, 6, and 9) PP2A in the presence (lane 12) or absence (lanes 1–11) of okadaic acid. The reaction mixtures were subjected to SDS-PAGE and immunoblotted with αPY (lanes 1–5) or αSH2-B (lanes 11 and 12). The blot corresponding to lanes 1–5 was stripped and reprobed with αSH2-B (lanes 6–10). The migration of molecular weight standards (×10^-3), p84, PDGFR, and SH2-Bβ is indicated.

PDGF-BB Promotes Phosphorylation of SH2-Bβ at Multiple Sites—To provide evidence that the multiple proteins recognized by αSH2-B in PDGF-treated cells reflect different phosphorylation states of SH2-Bβ, αSH2-B immunoprecipitates were treated with alkaline phosphatase in the presence or absence of sodium vanadate, an inhibitor of alkaline phosphatase. As discussed above, PDGF-BB treatment decreased the migration of SH2-Bβ (Fig. 5A, lane 2). Alkaline phosphatase treatment reduced the multiple forms of SH2-Bβ observed in PDGF-BB-treated cells (Fig. 5A, lane 2) to a faster migrating form (Fig. 5A, lane 3). Simultaneously, the intensity of the faster migrating form of SH2-Bβ increased significantly (Fig. 5A, lane 3), indicating a shift of SH2-Bβ from slower to faster migrating forms. Sodium vanadate significantly reduced the effect of alkaline phosphatase on the PDGF-BB-induced mobility shift of SH2-Bβ (Fig. 5A, lane 4), indicating that the change in migration of SH2-Bβ by alkaline phosphatase is due to dephosphorylation.

To provide evidence for the multiple bands in the presence of PDGF being due at least in part to serine and/or threonine phosphorylation, cell lysates from PDGF-BB-treated 3T3-F442A cells were incubated with αSH2-B. αSH2-B immunoprecipitates were treated with PP2A (a serine/threonine-specific phosphatase) and immunoblotted with αPY (Fig. 5B, lanes 1–5) and subsequently with αSH2-B (Fig. 5B, lanes 6–12). Consistent with our previous report (1), PP2A treatment of proteins from control cells reduced the multiple forms of SH2-Bβ to a single faster migrating form (Fig. 5B, lanes 6–8). Interestingly, in PDGF-BB-stimulated cells, PP2A treatment reduced the broad, diffuse bands of SH2-Bβ (Fig. 5B, lane 9) to two distinct, faster migrating protein bands (Fig. 5B, lanes 10 and 11). This condensation of bands is consistent with SH2-Bβ being phosphorylated on multiple serines/threonines in the presence of PDGF. The two bands observed with PP2A treatment aligned exactly with the two major tyrosyl-phosphorylated SH2-Bβ bands observed in the corresponding αPY blot (Fig. 5B, lane 5), suggesting that PDGF stimulates the phosphorylation of at least 2 tyrosines within SH2-Bβ. Okadaic acid, a potent inhibitor of PP2A, completely abrogated the effect of PP2A on the PDGF-induced mobility shift of SH2-Bβ (Fig. 5B, lane 12). Although PP2A increased substantially the migration of tyrosyl-phosphorylated SH2-Bβ (Fig. 5B, lane 5), it did not affect the degree of tyrosyl phosphorylation of either SH2-Bβ or SH2-Bβ-associated PDGFR (Fig. 5B, lanes 4 and 5), indicating that it is specific for serines/threonines as expected.

Multiple Tyrosyl-phosphorylated Proteins Associate with SH2-Bβ in Response to PDGF—A tight, tyrosyl-phosphorylated protein band designated p84 coimmunoprecipitated with SH2-Bβ (Figs. 4, 5B, 6, and 7). Interestingly, p84 aligns with neither of the two forms of SH2-Bβ after dephosphorylation by PP2A (Fig. 5B, lanes 5, 10, and 11), suggesting that p84 is an SH2-Bβ-interacting protein and not a form of SH2-Bβ itself. PDGF stimulation increased the amount of tyrosyl-phosphorylated p84 associated with SH2-Bβ (Fig. 4, upper panel; Fig. 6, lane 4). p84 does not appear to associate with SH2-Bβ via PDGFR because αPDGFR did not immunoprecipitate p84 (Fig. 6, lane 2). Furthermore, when αSH2-B immunoprecipitates were first dissociated by boiling in SDS-containing buffer, and then re-immunoprecipitated with αSH2-B, p84, like PDGFR, did not coimmunoprecipitate with SH2-Bβ (Fig. 6, lane 5). These data indicate that αSH2-B does not cross-react with either PDGFR or p84, and that both PDGFR and p84 associate with SH2-Bβ in cells stimulated with PDGF. In addition to PDGFR, SH2-Bβ, and p84, multiple other tyrosyl-phosphorylated proteins were present in αSH2-B immunoprecipitates when cells were treated with PDGF-BB (Fig. 4, lanes 2–6, upper panel; Fig. 6, lane 4). Because tyrosyl-phosphorylated proteins of similar size are also precipitated by αPDGFR and PDGFR coimmunoprecipitates with SH2-Bβ, it is not clear whether these other phosphoproteins associate with SH2-Bβ directly or indirectly through their interaction with SH2-Bβ-
bound PDGFR. It is also unclear whether PDGFR stimulates the association of p84 and/or these other phosphoproteins with SH2-Bβ or SH2-Bβ constitutively associates with these proteins and PDGFR stimulates their tyrosyl phosphorylation.

**PDGFR-AA and Epidermal Growth Factor (EGF) Stimulate Phosphorylation and a Shift in Mobility of SH2-Bβ—**

As PDGFR-BB is able to activate both α and β subunits of PDGFR, it is not clear which subunit in 3T3-F442A fibroblasts recruit and phosphorylate SH2-Bβ in response to PDGFR-BB. To begin to dissect which subunit of PDGFR utilizes SH2-Bβ, 3T3-F442A cells were treated with PDGFR-AA (which activates only the α subunit of PDGFR, Ref. 17), and solubilized proteins were immunoprecipitated with αSH2-B and immunoblotted with αPY. The extent of ligand-induced tyrosyl phosphorylation of SH2-Bβ and the multiple other proteins including PDGFR that coimmunoprecipitate with SH2-Bβ was similar between cells stimulated with PDGFR-AA and PDGFR-BB (Fig. 7, lanes 2 and 3), with PDGFR-AA being a little less effective than PDGFR-BB at the same dosage. PDGFR-AA, like PDGFR-BB, caused a decrease in SH2-Bβ mobility (Fig. 7, lanes 7 and 8). These data suggest that the α subunit of PDGFR recruits SH2-Bβ as a signaling protein in 3T3-F442A cells. Whether SH2-Bβ also associates with PDGFRβ remains to be determined.

To investigate whether other receptor tyrosine kinases regulate SH2-Bβ, 3T3-F442A cells, which are also responsive to EGF (10), were stimulated with 100 ng/ml EGF for 10 min, and αSH2-B immunoprecipitates were immunoblotted with αPY. A tyrosyl-phosphorylated protein of a size appropriate for EGF receptor coimmunoprecipitated with SH2-Bβ from EGF-treated (Fig. 7, lane 5) but not control (Fig. 7, lane 1) cells, suggesting that SH2-Bβ is present in a complex with EGF receptor. Surprisingly, tyrosyl phosphorylation of SH2-Bβ was not detectable using αPY (Fig. 7, lane 5), although a significantly reduced migration rate of SH2-Bβ was observed in EGF-treated cells (Fig. 7, lane 10). Similarly, EGF stimulated a large shift in mobility of SF-2-B in PC12 cells without detectable tyrosyl phosphorylation (data not shown). Incubation of αSH2-B immunoprecipitates with alkaline phosphatase completely abolished the EGF-induced mobility shift (data not shown). These data suggest that the EGF-induced mobility shift is most likely due to phosphorylation, and that the phosphorylation of SH2-Bβ elicited by EGF occurs mainly on serines and/or threonines.

The proposal that EGF, as well as PDGFR-BB, stimulates phosphorylation of SH2-Bβ on serines/threonines is further suggested by the finding that EGF and PDGFR-BB stimulate tyrosyl phosphorylation of SH2-Bβ to a much lesser extent than GH (Fig. 7, lanes 3–5) but cause a greater (PDGFR-BB) or similar (EGF) shift in mobility of SH2-Bβ (Fig. 7, lanes 8–10).

**DISCUSSION**

In the current study, we show that SH2-Bβ binds directly to tyrosyl-phosphorylated, but not unphosphorylated, PDGFR in both GST fusion protein pull-down and Far Western blotting assays. The SH2 domain of SH2-Bβ is sufficient for binding to PDGFR in the GST fusion protein pull-down assay. Furthermore, when the conserved Arg was mutated to Glu within the FLVRQS motif in the SH2 domain of SH2-Bβ, the ability of mutant SH2-Bβ to bind PDGFR was dramatically inhibited. These results suggest that the SH2 domain of SH2-Bβ is both necessary and sufficient for binding to tyrosyl-phosphorylated, activated PDGFR. The ligand-dependent interaction of SH2-Bβ with PDGFR was further confirmed by the coimmunoprecipitation of endogenous SH2-Bβ with endogenous PDGFR in both 3T3-F442A and NIH3T3 cells.

The finding that there is an upward shift in mobility of SH2-Bβ upon PDGFR-BB treatment that is abolished by alkaline phosphatase provides clear evidence that PDGFR promotes phosphorylation of SH2-Bβ. Similarly, blotting with αPY provides strong evidence that PDGFR promotes tyrosyl phosphorylation of SH2-Bβ. The fact that PPP2A condenses the broad SH2-Bβ band to two faster migrating bands suggests that at least two tyrosines are phosphorylated. Because SH2-Bβ binds directly to activated PDGFR, it is logical to hypothesize that SH2-Bβ is phosphorylated directly by PDGFR. In support of this, when coexpressed in COS cells, SH2-Bβ is tyrosyl-phosphorylated by PDGFR β subunit.2

The fact that PPP2A increases the migration of SH2-Bβ in control and PDGFR-treated cells suggests that SH2-Bβ is phosphorylated on serines and/or threonines. In support of PDGFR and/or EGF stimulating the serine/threonine phosphorylation of SH2-Bβ, there is a discrepancy between changes in SH2-Bβ mobility and amount of αPY binding to SH2-Bβ. A maximal decrease in mobility of SH2-Bβ occurs at shorter times and at lower PDGFR-BB concentrations than the maximal increase in tyrosyl phosphorylation as detected by αPY. In the extreme case of EGF, no signal is detectable by αPY but a significant decrease in SH2-Bβ mobility is observed. In addition,

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2 L. Rui, A. Kazlauskas, and C. Carter-Su, unpublished data.
PDGF-BB stimulates a greater decrease in SH2-Bβ mobility than GH, but is much less effective than GH at stimulating tyrosyl phosphorylation of SH2-Bβ. Although we favor the hypothesis that PDGF and EGF stimulate the serine/threonine phosphorylation of SH2-Bβ, our data do not exclude the possibility that SH2-Bβ is constitutively phosphorylated on serines/threonines and that EGF, PDGF, and GH stimulate the phosphorylation of different tyrosines on SH2-Bβ. However, one would have to hypothesize that these tyrosines phosphorylated by EGF receptor are not recognized by the αPY used in this study, that those phosphorylated by PDGFR include some that are recognized by αPY and some that are not, and that those phosphorylated in response to GH bind αPY with high affinity. It would not be surprising for SH2-Bβ to be phosphorylated on multiple serines/threonines because sequence analysis reveals that SH2-Bβ has 82 serines and 26 threonines, including multiple potential phosphorylation sites for protein kinase C and a potential site (PLSP) for mitogen-activated protein kinases (e.g. ERK1/2). Protein kinase Cs and/or ERKs are potential candidates for PDGF-induced serine/threonine phosphorylation of SH2-Bβ, because PDGF is reported to activate multiple isoforms of protein kinase C (18–22) and ERKs 1 and 2 (23).

We observed a tyrosyl-phosphorylated protein with $M_r \sim 84,000$ (p84) coimmunoprecipitating with SH2-Bβ in PDGF-stimulated cells. When the αSH2-B immunocomplex was dissociated by boiling in SDS-containing buffer, p84 was no longer immunoprecipitated by αSH2-B, suggesting that αSH2-B interacts with p84 indirectly through SH2-Bβ rather than directly binding to p84. p84 does not coimmunoprecipitate with PDGFR, suggesting that the interaction of SH2-Bβ with p84 is not mediated by PDGFR. The identity of p84 is not known. It is unlikely that p84 is the p85 subunit of phosphatidylinositol 3’-kinase because p84 is not recognized by anti-p85 in immunoblots (data not shown). Interestingly, when αSH2-B raised from a different rabbit (rabbit 2) was used to immunoprecipitate SH2-Bβ, another tyrosyl-phosphorylated protein with $M_r \sim 145,000$ (p145) was observed in αSH2-B immunoprecipitates only from PDGF-stimulated cells (data not shown). We therefore believe that SH2-Bβ interacts with multiple proteins besides PDGFR, as expected for an adapter protein involved in PDGFR signaling. SH2-Bβ thereby may actively regulate PDGFR signaling by initiating some as yet unidentified pathways.

PDGF-induced phosphorylation of SH2-Bβ may play a significant role in PDGFR signaling. The phosphorylated tyrosines in SH2-Bβ may form docking sites for other signaling molecules which contain SH2 or phosphotyrosine binding domains, which may include p84 and p145 as discussed above. The significance of serine and/or threonine phosphorylation of SH2-Bβ is unclear. Phosphoserine(s)/threonines in SH2-Bβ could serve as a binding site for other signaling molecules such as 14-3-3 (24–29). Serine/threonine phosphorylation of SH2-Bβ could also inhibit tyrosine phosphorylation of SH2-Bβ, as reported for insulin receptor substrate-1 (30, 31), or affect the association of SH2-Bβ with other signaling molecules, as reported for αsos association with Grb2 (11, 12, 32, 33). Two isoforms of SH2-B, designated SH2-Bo and SH2-Bβ, have been described to date (1, 34). SH2-B, along with Lnk and APS, are proposed to form a new adapter family (35). Lnk, with an SH2 domain 68% identical to SH2-B, is expressed preferentially in lymphoid tissues and has been shown to bind to phosphatidylinositol 3’-kinase, Grb2, and phospholipase Cγ (36). APS, with a PH domain 58% identical to that of SH2-B and an SH2 domain 80% identical to that of SH2-B, was cloned as a binding protein for the kinase domain of c-KIT receptor and is predicted to play a role in B cell antigen receptor activation (35). As the SH2 domain of SH2-Bβ, which is highly conserved among SH2-Bβ, Lnk and APS, mediates the interaction between SH2-B and PDGFR, we predict that Lnk, APS, SH2-Bo, or their homologues also bind to activated PDGFR and serve as signaling molecules for PDGFR in those cells that express both PDGFR and the SH2-B-related proteins.

In summary, we have shown that in response to PDGF, SH2-Bβ is recruited onto PDGFR complexes via direct interaction with PDGFR, and that tyrosyl-phosphorylated SH2-Bβ is also phosphorylated on serines/threonines. Serine/threonine phosphorylation of SH2-Bβ appears to be increased by PDGF and EGF stimulation. The SH2 domain of SH2-Bβ is required and sufficient for the interaction of SH2-Bβ with tyrosyl-phosphorylated PDGFR. As a consequence of association of SH2-Bβ with PDGFR, signaling molecules bound to SH2-Bβ such as p84 are also recruited by PDGFR. We conclude that SH2-Bβ is a previously unknown signaling molecule for PDGFR signaling. It will be interesting to determine whether SH2-Bβ mediates some of the actions of PDGF that cannot be accounted for by previously identified PDGFR signaling molecules.

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