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Phosphorylation of Tyrosine 720 in the Platelet-Derived Growth Factor α Receptor Is Required for Binding of Grb2 and SHP-2 but Not for Activation of Ras or Cell Proliferation

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Following binding of platelet-derived growth factor (PDGF), the PDGF α receptor (αPDGFR) becomes tyrosine phosphorylated and associates with a number of signal transduction molecules, including phospholipase Cγ-1 (PLCγ-1), phosphatidylinositol 3-kinase (PI3K), the phosphotyrosine phosphatase SHP-2, Grb2, and Src. Here, we present data identifying a novel phosphorylation site in the kinase insert domain of the αPDGFR at tyrosine (Y) 720. We replaced this residue with phenylalanine and expressed the mutated receptor (F720) in Patch fibroblasts that do not express the αPDGFR. Characterization of the F720 mutant indicated that binding of two proteins, SHP-2 and Grb2, was severely impaired, whereas PLCγ-1 and PI3K associated to wild-type levels. In addition, mutating Y720 to phenylalanine dramatically reduced PDGF-dependent tyrosine phosphorylation of SHP-2. Since Y720 was required for recruitment of two proteins, we investigated the mechanism by which these two proteins associated with the αPDGFR. SHP-2 bound the αPDGFR directly, whereas Grb2 associated indirectly, most probably via SHP-2, as Grb2 and SHP-2 coimmunoprecipitated when SHP-2 was tyrosine phosphorylated. We also compared the ability of the wild-type and F720 αPDGFRs to mediate a number of downstream events. Preventing the αPDGFR from recruiting SHP-2 and Grb2 did not compromise PDGF-AA-induced activation of Ras, initiation of DNA synthesis, or growth of cells in soft agar. We conclude that phosphorylation of the αPDGFR at Y720 is required for association of SHP-2 and Grb2 and tyrosine phosphorylation of SHP-2; however, these events are not required for the αPDGFR to activate Ras or initiate a proliferative response. In addition, these findings reveal that while SHP-2 binds to both of the receptors, it binds in different locations: to the carboxy terminus of the βPDGFR but to the kinase insert of the αPDGFR.

Platelet-derived growth factor (PDGF) was discovered as a potent mitogen and chemotactic agent for cells of connective-tissue origin. It consists of a homo- or heterodimer of two homologous chains, A and B, which are the products of distinct genes. All three pairwise combinations of PDGF exist and are biologically active (9). Receptors for PDGF (PDGFRs) were originally found on mesenchymal cells, such as fibroblasts, vascular smooth muscle cells, and glial cells, and have more recently been detected on neuronal, epithelial, and endothelial cells (5, 14, 19, 21–23, 51, 67). Binding of PDGF drives dimerization of the PDGFR subunits, of which there are two types, α and β. The β subunit preferentially binds the B chain of PDGF, whereas the α subunit binds both A and B chains with high affinity. As a result, PDGF-AA drives formation of αα homodimers, PDGF-AB induces αα homodimers and αβ heterodimers, and PDGF-BB assembles all possible PDGFR subunit dimers: αα, αβ, and ββ (9).

Activation of the αPDGFR triggers a number of biological responses which in normal cells includes progression through the cell cycle and cellular proliferation. When deregulated, these events have pathological consequences, and the αPDGFR appears to play an important role in the development of certain types of tumors. For instance, increased expression of the αPDGFR correlates with an elevation of metastatic potential of cell lines derived from lung tumors and ovarian carcinomas (13, 19, 22). Furthermore, expression of the αPDGFR serves as a molecular marker for malignant astrocytomas, and establishment of autocrine loops involving the αPDGFR and PDGF-AA may be the underlying cause of the hyperproliferative state (38, 39, 50, 61).

In addition to its role in tumors, the αPDGFR also plays an important role in mouse embryogenesis. PDGF-AA and the αPDGFR are coexpressed in preimplantation embryos, and following postimplantation, their expression becomes confined to separate regions of the embryo (41). These observations suggest that PDGF-AA drives certain events during embryogenesis, and also that there is a shift from an autocrine to a paracrine relationship during the development of the mouse embryo (41). Furthermore, expression of PDGF-AA mRNA in neurons and the αPDGFR message in glial cells has been shown to be developmentally regulated (66, 67). A series of elegant studies examining the role of PDGF-AA (and thus the αPDGFR) in the development of the rat optic nerve indicate that PDGF-AA regulates differentiation of the O2A progenitor cells and acts as a survival factor (2, 40, 44, 45). Convincing support of the idea that the αPDGFR is important during embryogenesis is the observation that deletion of αPDGFR in the *Patch* mouse results in a variety of disorders, including embryonic lethality at approximately E9.5 (37, 47, 56). Finally, mice that do not have the gene for the PDGF-A chain either die in utero or survive to be born but develop lung emphysema arising at least in part from the absence of alveolar myofibro-

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blasts (8). Together these studies strongly indicate that the αPDGFR is vital for proper embryogenesis.

The βPDGFR is also essential for embryonic development; however, deletion of the βPDGFR leads to a phenotype that is distinct from that of the *Ph*/*Ph* mouse. Mice homozygous for the βPDGFR deletion die at or shortly after birth, are anemic, hemorragic, and thrombocytopenic, and are unable to form glomeruli in the kidneys (55). The distinct phenotype of mice in which the PDGFRs have been individually eliminated is consistent with the possibility that the two receptors have distinct roles during the formation of a mouse embryo.

There are numerous explanations for the underlying basis of the distinct phenotypes of the *Ph*/*Ph* and βPDGFR knockout mice, and one possibility is that the two PDGFRs initiate distinct biological responses. While both of the receptors are able to initiate DNA synthesis and cell proliferation, there have been reports that the α- and βPDGFRs do not initiate identical biological responses. For instance, the αPDGFR promotes cellular hypertrophy in vascular smooth muscle cells, whereas DNA synthesis is mediated only by PDGF-BB (24). In addition, the two receptors differ in their ability to initiate several biological responses in certain cell types (4, 16, 62, 68). The basis of these differences may lie in the signal relay pathways engaged by each of the PDGFRs, and considerable effort has been directed towards elucidating these events.

Most of the information regarding signal transduction by the PDGFR comes from studies on the βPDGFR (for reviews, see references 9 and 26); however, as the signaling cascades employed by the αPDGFR begin to emerge, it appears that there are fundamental differences between signal transduction pathways initiated by the two PDGFRs. As in the case of the βPDGFR, ligand binding induces dimerization of the αPDGFR subunits and results in phosphorylation of the receptor at multiple tyrosine residues. As a result, the αPDGFR associates with numerous SH2 domain-containing proteins, including phospholipase Cγ-1 (PLCγ-1), the regulatory subunit (p85) of the phosphatidylinositol 3-kinase (PI3K), the phosphotyrosine phosphatase SHP-2 (previously called SH-PTP2, PTP-1D, and Syp), Grb2, Src, and an as-yet-unidentified 120-kDa protein (3, 69). Unlike the βPDGFR, the αPDGFR does not associate with or trigger tyrosine phosphorylation of the GTPase-activating protein of Ras RasGAP) (3, 20). Thus, although both PDGFRs become tyrosine phosphorylated and associate with SH2-containing signaling enzymes, different sets of signal transduction molecules bind to each of the PDGFRs.

An additional difference is the relative importance of these receptor-associated proteins in relaying the receptor's mitogenic signal. While PI3K, PLCγ, and Src have been shown to be required for initiation of DNA synthesis by the βPDGFR, the αPDGFR does not seem to require either PI3K or PLCγ for mitogenic signaling (15, 17, 30, 59, 60, 68, 69). The involvement of Src in αPDGFR signal relay has not yet been investigated. These studies show that tyrosine phosphorylation of the αPDGFR leads to stable association with numerous signaling molecules; however, which (if any) of these associated proteins are the αPDGFR's effectors remains unknown. Identification of the αPDGFR tyrosine phosphorylation sites is likely to lead to additional insights into how the various proteins associate with the αPDGFR and their relative importance in αPDGFR-mediated signaling.

Here we report that one of the αPDGFR tyrosine phosphorylation sites is at position Y720 in the kinase insert. Replacement of this tyrosine with a phenylalanine abolished PDGF-dependent binding of SHP-2 and Grb2 to the αPDGFR, tyrosine phosphorylation of SHP-2, and assembly of a complex between SHP-2 and Grb2. Surprisingly, these events were not required for initiating Ras activation and DNA synthesis, indicating that PDGF AA-mediated Ras activation does not require Grb2 binding and that DNA synthesis can be triggered without tyrosine phosphorylation or stable binding of SHP-2. Furthermore, the observation that SHP-2 binds to the αPDGFR within the kinase insert reveals an intriguing difference between the α- and βPDGFRs, since SHP-2 binds to the C-terminal tail of the βPDGFR.

MATERIALS AND METHODS

Cell lines. The mouse embryo 3T3 Patch B (PhB) cell line was derived from *Ph*/*Ph* mouse embryos as described previously (49), and they were kindly provided by Dan Bowen-Pope. They were maintained in Dulbecco's modified Eagle's (DME) medium supplemented with 10% fetal bovine serum (FBS). The wild-type (WT) and F720 human αPDGFR cDNAs were subcloned into the pLXSHD retroviral vector (57) and expressed in PhB cells, as previously described (27). Briefly, DNA was transfected into Ψ2 cells, and the viral supernatant from these cells was used to infect PA317 cells. Virus from mass populations of histidinol-resistant PA317 cells was used to infect PhB cells, which were then selected on the basis of resistance to 4 mM histidinol. Mass populations of drug-resistant PhB cells were used for the subsequent studies.

Antibodies. The RasGAP antiserum (70.3) used in these studies was a crude polyclonal that has been previously described (59). The αPDGFR antibodies (27 and 29) were crude rabbit polyclonals raised against a glutathione *S*-transferase (GST) fusion protein including either the carboxy terminus (amino acids 951 to 1089) or the kinase insert (amino acids 671 to 789) of the human αPDGFR, respectively. The αPDGFR antisera recognize the mouse and human αPDGFRs and do not cross-react with the βPDGFR of either species. The PLC-γ1, PI3K, Grb2, and 4G10 antiphosphotyrosine were purchased from Upstate Biotechnology, Inc., and used according to the manufacturer's specifications. The antiphosphotyrosine antibody PY20 was purchased from ICN and used according to the manufacturer's specifications. The Ras antibody was Y13-259, a rat monoclonal that was purified on a protein G column and kindly provided by Ann Harwood. The SHP-2 antibodies used for Western blotting were a 1:1 mixture of anti-Syp (29) (provided by Gen-Sheng Feng) and a crude polyclonal raised against a GST fusion including the last 44 amino acids of human SHP-2. Both of the antisera were diluted 1:1,000 for Western blotting.

Radiolabeling of the PDGFR, SHP-2, and fusion protein. To radiolabel the PDGFR or SHP-2 in intact cells, PhB cells were grown to confluence, then starved for 24 h in DME medium plus 0.1% calf serum (CS), and incubated for 24 h in modified Eagle's medium (MEM) lacking phosphate and containing 0.1% CS (LM). They were incubated in LM containing 5 mCi of $^{32}P_i$ per ml for 4 h at 37°C, and the cells were then exposed to buffer (10 mM acetic acid plus 2 mg of bovine serum albumin (BSA) per ml) or to 50 ng of PDGF-AA per ml for 5 min at 37°C; the cells were lysed in EB (10 mM Tris-HCl [pH 7.4], 5 mM EDTA, 50 mM NaCl, 50 mM NaF, 0.1% BSA, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 2 mM Na_3VO_4, and 20 μg of aprotinin per ml), and the PDGFR or SHP-2 was immunoprecipitated. To label the PDGFR in vitro, receptor immunoprecipitates prepared from either resting or stimulated cells were subjected to an in vitro kinase assay. To radiolabel the kinase insert GST fusion protein, 1 μg of purified (59) fusion protein was subjected to a standard in vitro kinase assay in the presence of PDGFR immunoprecipitates.

Phosphopeptide maps. Immunoprecipitates of radiolabeled αPDGFR or SHP-2 were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 7.5% gel, visualized by autoradiography, recovered from the gel, trichloroacetic acid precipitated, performic acid oxidized, and digested exhaustively with trypsin followed by thermolysin. The resulting peptides were resolved by thin-layer electrophoresis (TLE) at pH 8.9 (αPDGFR) or at pH 1.9 (SHP-2) and then by ascending chromatography (isobutyric acid-pyridine-glacial acetic acid-water-butanol, 65:5:3:29:2) as described previously (25).

Site-directed mutagenesis. The 1.7-kb *Pst*I-*Bam*HI fragment of the human αPDGFR was subcloned into the pBS^+ plasmid, and the resulting construct was called 19E. Site-directed mutagenesis was carried out by using the Amersham oligonucleotide-directed in vitro mutagenesis kit. The following oligonucleotide was used to mutate Y720 to phenylalanine: 5′GATGAAAGCACGCGTAGCT TTGTTATTTTA3′. This oligonucleotide also introduced an *Mlu*I site without altering the amino acid sequence. Mutants were initially identified by restriction enzyme digestion with *Mlu*I and then verified by sequencing. The *Pst*I-*Bam*HI fragment of 19E was then subcloned back into 18F, which includes the entire αPDGFR cDNA as a 3.5-kb *Not*I-*Bam*HI insert in $pSKII^+$ vector. Finally, the *Not*I-*Bam*HI fragment was subcloned into the pLXSHD retroviral vector.

Immunoprecipitation. PhB cells expressing the empty vector or the αPDGFR were grown to confluence, incubated overnight in DME medium containing 0.1% CS, and lysed in EB; 1 μl of 27P was added to the lysate of 2×10^6 cells, and the mixture was incubated on ice for 1.5 h. Immune complexes were collected on a suspension of formalin-fixed *Staphylococcus aureus*, spun through a 600-μl cushion of EB plus 10% sucrose, washed twice with 1.0 ml of RIPA buffer (150 mM NaCl, 10 mM $NaPO_4$, 2 mM EDTA, 1% sodium deoxycholate, 1% Nonidet P-40 [NP-40], 0.1% SDS, 20 μg of aprotinin per ml, 50 mM NaF, 2 mM

Na_3VO_4, 0.1% 2-mercaptoethanol), washed twice with 1.0 ml of PAN {10 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid), pH 7.0], 100 mM NaCl, 20 μg of aprotinin per ml}, plus 0.5% NP-40, washed twice with 1.0 ml of PAN, and finally resuspended in PAN and stored at −70°C.

Western blot analysis. The αPDGFR, Grb2, or SHP-2 was immunoprecipitated from resting or PDGF-stimulated cells (50 ng of PDGF-AA per ml for 5 min at 37°C), immunoprecipitates from 3×10^6 cells were resolved on an SDS–7.5% PAGE gel, and the proteins were transferred to Immobilon. The membranes were incubated for an hour at room temperature in either Block (10 mM Tris-Cl [pH 7.5], 150 mM NaCl, 10 mg of BSA per ml, 10 mg of ovalbumin per ml, 0.05% Tween 20, 0.005% NaN_3) for antiphosphotyrosine Western blots or Blotto [10 mM Tris-HCl (pH 7.5) 150 mM NaCl, 10 mg of nonfat dry milk per ml, 0.05% Tween 20, 0.005% NaN_3] for all other Western blots. The membranes were further incubated for 2 h at room temperature with a primary antibody diluted in Blotto 1/5,000 (RasGAP 70.3) or 1/2,000 (αPDGFR antibody 29), while the PLC-γ1 and PI3K antibodies were diluted as recommended by the manufacturer. For antiphosphotyrosine Western blots, a mixture of antiphosphotyrosine antibodies was used at a dilution of 1 μg of PY20 and 2 μg of 4G10 per ml in Block. The membranes were then incubated with an alkaline phosphatase-conjugated goat anti-mouse or goat anti-rabbit secondary antibody diluted 1/2,000 in Blotto. Finally, a solution of 0.165 mg of BCIP (5-bromo-4-chloro-3-indolylphosphate *p*-toluidine salt) per ml and 0.33 mg of NBT (*p*-nitroblue tetrazolium chloride) per ml in 0.1 M Tris-HCl (pH 9.5)–0.1 M NaCl–0.05 M $MgCl_2$ was added to develop the Western blot. In some experiments the secondary antibody was conjugated to horseradish peroxidase, and the enhanced chemiluminescence kit (Amersham) was used as the detection system.

In vitro kinase assay. Immunoprecipitates were incubated with 20 mM PIPES (pH 7.0)–10 mM $MnCl_2$–20 μg of aprotinin per ml–10 μCi of [γ-^{32}P]ATP for 10 min at 30°C, in a total reaction volume of 20 μl. The reaction was stopped by adding 20 μl of 2× sample buffer (10 mM EDTA, 4% SDS, 5.6 M 2-mercaptoethanol, 20% glycerol, 200 mM Tris-HCl [pH 6.8], 1% bromophenol blue). The samples were then incubated for 4 min at 98°C and spun in a Savant HSC 10K Speedfuge at 8,000 rpm for 5 min, and the supernatant was resolved on a SDS–7.5% PAGE gel.

[^{3}H]thymidine uptake. PDGF-stimulated incorporation of [^{3}H]thymidine was assayed as follows. Cells were plated at 8×10^4/ml in DME medium plus 0.5% CS in 24-well dishes. After 3 days, the cells were washed twice with 1 ml of phosphate-buffered saline (PBS) and placed in 0.5 ml of DME medium, containing 2 mg of BSA per ml, for 2 days. PDGF buffer (10 mM acetic acid, 2 mg of BSA per ml), 10% fetal bovine serum, or different concentrations of PDGF-AA or 50 ng of PDGF-BB per ml was added. The cultures were incubated for 18 h, the medium was replaced with 0.5 ml of DME medium plus 5% CS containing 0.8 μCi of [^{3}H]thymidine per ml, and the incubation was prolonged for 4 h. Cells were then washed with ice-cold 5% trichloroacetic acid and lysed in 0.25 N NaOH, and the trichloroacetic acid-precipitable material was harvested and quantitated in the presence of scintillation fluid.

Soft-agar assay. Six-well tissue culture plates were coated with a layer of DME medium plus 5% CS containing 0.6% SeaPlaque agarose (FMC Bioproducts, Rockland, Maine). Subconfluent cells were trypsinized, washed twice in PBS, and resuspended in DME medium plus 5% CS at 10^5 cells per ml. One milliliter of this cell suspension was added to 1 ml of DME medium plus 5% CS plus 0.9% low-melting-point agarose containing 200 ng of PDGF-AA per ml or PDGF buffer, and the cells were plated onto the coated tissue culture plates and placed in a 37°C incubator. After 10 days, colony formation was analyzed with an inverted microscope.

Activation of Ras. Ras activation assays were performed as described by Satoh et al. (46). Briefly, confluent 5-cm-diameter dishes of cells were incubated in DME medium plus 0.1% CS for 48 h and then in MEM lacking phosphate and containing 2 mg of BSA per ml for 24 h, after which the medium was aspirated and replaced by 2 ml of fresh MEM lacking phosphate, containing 2 mg of BSA per ml, and containing 0.25 μCi of $^{32}P_i$ per ml, and the incubation was extended for 4 h at 37°C. The cells were left resting or were stimulated with 50 ng of PDGF-AA per ml for 5 min at 37°C, placed on ice, washed three times with ice-cold Tris-buffered saline (TBS; 25 mM Tris-HCl [pH 7.4], 137 mM NaCl, 5 mM KCl, 0.7 mM $CaCl_2$, 0.5 mM $MgCl_2$), and then lysed in 0.2 ml of lysis buffer (TBS containing 1% NP-40, 10 μg of aprotinin per ml, 1 mM phenylmethylsulfonyl fluoride, 10 μg of leupeptin per ml, 16 mM $MgCl_2$, and 10 μg of Y13-259 anti-Ras antibody). The cells were dislodged by scraping and then transferred to a microcentrifuge tube, the dishes were rinsed with 0.3 ml of lysis buffer without antibody, and this solution was added to the cell lysate. The insoluble material was pelleted by centrifugation at 12,000 × *g* for 30 min, and the supernatant was transferred to a new microcentrifuge tube and incubated on ice for 2 h, after which 15 μl of pelleted goat anti-rat immunoglobulin G agarose beads was added and the tubes were incubated end over end for 1 h at 4°C. The beads were washed eight times with TBS containing 1% NP-40 and 16 mM $MgCl_2$, and the immunoprecipitated proteins were released from the beads by addition of 25 μl of elution buffer (2 mM EDTA, 0.2% SDS, 2 mM dithiothreitol, 0.5 mM GTP, and 0.5 mM GDP) and incubation at 98°C for 3 min. The beads were pelleted, and the supernatant was spotted on a polyethyleneimine cellulose plate with fluorescent indicator and chromatographed in a buffer of 0.75 M KH_2PO_4. The positions of GDP and of GTP standards were visualized by UV light, and the radioactivity was quantitated by PhosphorImager analysis.

RESULTS

Identification of tyrosine 720 as a phosphorylation site in the kinase insert domain of the αPDGFR. To identify the tyrosine phosphorylation sites of the αPDGFR, PhB cells expressing the WT human αPDGFR were incubated with $^{32}P_i$ for 4 h, and then for an additional 10 min with or without PDGF-AA. The cells were lysed, and the αPDGFR was immunoprecipitated with an αPDGFR-specific antibody. The immunoprecipitated proteins were resolved by SDS-PAGE, and the 170-kDa αPDGFR was excised from the gel and exhaustively digested with trypsin and then with thermolysin. The resulting phosphopeptides were resolved in two dimensions, and an autoradiogram of the thin-layer plate is shown in Fig. 1A and B. The numbered spots were present only in the PDGF-stimulated samples and contained exclusively phosphotyrosine (data not shown), revealing that the αPDGFR is phosphorylated at multiple tyrosine residues upon stimulation with PDGF-AA. We also examined the phosphorylation sites of the αPDGFR labeled in vitro. The αPDGFR was immunoprecipitated from PDGF-AA-stimulated cells and subjected to an in vitro kinase in the presence of [γ-^{32}P]ATP, and then the receptor was analyzed by two-dimensional mapping analysis (Fig. 1C). The in vitro-labeled receptor was exclusively phosphorylated on tyrosine residues (data not shown). Comparison of the tyrosine phosphorylation sites labeled in vivo and in vitro showed that most sites were phosphorylated under both conditions (Fig. 1D).

Having determined that the αPDGFR is phosphorylated at multiple tyrosine residues, we set out to identify these sites. Several of the major phosphotyrosine-containing peptides, 2, 4, and 8, were recovered from the thin-layer plate, individually subcleaved with four different proteases (*Pseudomonas fragi* protease, *S. aureus* V8, chymotrypsin, and metalloendopeptidase) as previously described (27), and then resolved by TLE or ascending chromatography. Sensitivity to a given protease was scored as a change in mobility relative to that of the undigested phosphopeptide, and it indicated whether the peptide contained a site for the proteases used (Table 1). This analysis gave us some idea of which amino acids are and are not present in the phosphotyrosine-containing peptides, and from the predicted amino acid sequence of the αPDGFR, we chose candidate peptides for spots 2, 4, and 8. Unfortunately, more than one candidate peptide was predicted for each of the phosphopeptide spots. Therefore, we simplified the task of mapping phosphorylation sites by analyzing smaller fragments of the receptor's intracellular domain. We made a fusion protein encoding only the kinase insert domain of the αPDGFR, phosphorylated it in an in vitro kinase reaction, and then subjected the phosphorylated fusion protein to phosphopeptide mapping as described above. As illustrated in Fig. 2, spots 1 through 5, 8, 10, 12, and 13 were common to both the kinase insert fusion protein and the intact receptor, indicating that some of the αPDGFR phosphorylation sites are in the kinase insert domain. Examination of the kinase insert sequence indicated that the tryptic-thermolytic Y720 dipeptide (SY) was the only possible tyrosine-containing peptide that matched the behavior of spot 8 in the subcleavage analysis (Table 1). In order to independently verify this conclusion, we analyzed spot 8 by manual Edman degradation and found that the phenylthiohydantoin-derivatized phosphotyrosine was liberated after the first cycle (data not shown). This demonstrated that the phosphorylated tyrosine is in the second position of the peptide and supports the possibility that the sequence of spot 8 is SY. Together, these data strongly indicated that spot 8 contains residue Y720. Similar studies revealed that spots 2 and 4

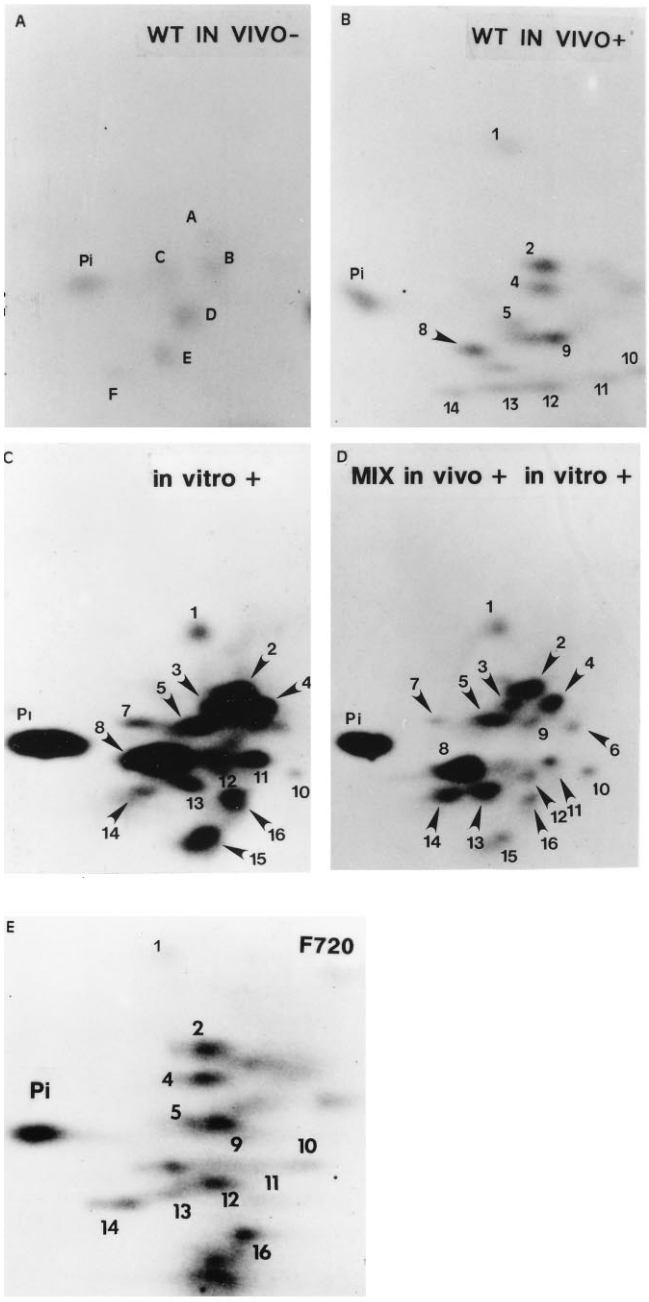

FIG. 1. Two-dimensional tryptic and thermolytic maps of the αPDGFR. Confluent, quiescent cells expressing the WT or F720 αPDGFR were labeled with $^{32}P_i$, exposed to buffer (−) or stimulated with PDGF-AA (+), and lysed, and the receptor was immunoprecipitated. Alternatively, the WT receptor was immunoprecipitated from PDGF-AA-stimulated cells and then labeled in an in vitro kinase reaction. The radiolabeled receptors were run on an SDS-PAGE gel, eluted from the gel, and digested with trypsin and thermolysin, and the resulting phosphopeptides were resolved by electrophoresis at pH 8.9 and then by chromatography. The panels are autoradiograms of the thin-layer plates. Panel E is a map of the F720 receptor isolated from metabolically labeled, PDGF-AA-stimulated cells. Spots labeled with letters are phosphoserine-containing peptides, whereas numbered spots contain phosphotyrosine. The arrowhead pointing to spot 8 in panel B indicates the position of the peptide containing tyrosine 720. Panel D shows a mixture of an equal number of counts of panels B and C. Pi, free phosphate.

TABLE 1. Subcleavage analysis of αPDGFR tryptic-thermolytic phosphopeptides

Protease	Amino acid(s) recognized	Susceptibility of phosphopeptide[a]:		
		2	4	8
P. fragi	C or D	+	+	−
Chymotrypsin	P, W, Y (not PY)	−	+	−
Metalloendopeptidase	K	−	−	−
S. aureus V8	E	−	−	−

[a] Phosphopeptides 2, 4, and 8 (resolved in Fig. 1C) were recovered from the TLE plate and subcleaved with the four proteases. The *P. fragii* protease and metalloendopeptidase cut on the N-terminal side of the indicated amino acid, whereas chymotrypsin and *S. aureus* V8 cleave on the C-terminal side of the amino acid. After cleavage, each sample was divided in two and resolved by TLE at pH 3.5 or ascending chromatography as described previously (27). A positive result was scored when the protease altered the mobility of the phosphopeptide relative to that of the undigested phosphopeptide.

were Y742-containing phosphopeptides and spots 12 and 13 were Y731-containing phosphopeptides (data not shown). Thus, it appears that the αPDGFR is phosphorylated at at least three tyrosine residues in the kinase insert, at positions 720, 731, and 742.

The above-described experiments convincingly implicated Y720 as a phosphorylation site; therefore, we mutated this tyrosine to phenylalanine, subcloned the mutant cDNA into the pLXSHD retroviral vector, and used the resulting virus to infect PhB cells, which lack endogenous αPDGFRs. Mass populations of drug-resistant cells were then tested for expression of the αPDGFR. Cells were grown to confluence and lysed, the insoluble material was removed by centrifugation, and the supernatant was analyzed by Western blotting with α- and βPDGFR-specific antisera. The parental cells contained no αPDGFR protein, whereas the WT and the F720 receptors were expressed to similar levels (Fig. 3A), which was approximately 60% of the level of the endogenous mouse βPDGFR (3). Western blot analysis of the three cell lines also indicated that they expressed similar levels of the endogenous mouse βPDGFR (Fig. 3A). Probing the same samples with anti-Ras Gap antibodies demonstrated that a comparable amount of cell lysate was present in each lane (Fig. 3A).

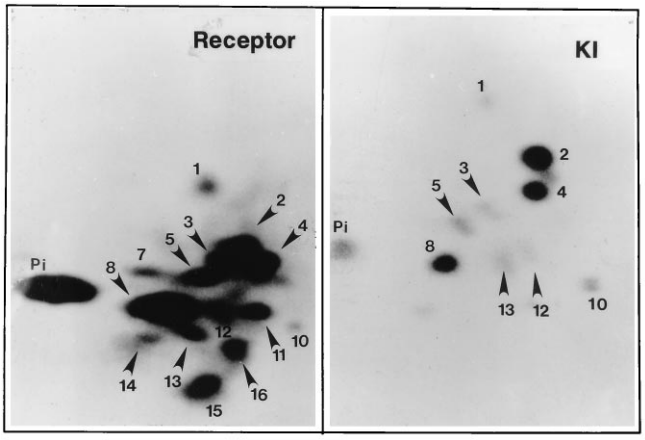

FIG. 2. Phosphopeptide maps of the intact receptor and the kinase insert fusion protein. The left panel shows a tryptic-thermolytic map of the in vitro-labeled WT receptor, and the right panel displays a map of the GST-kinase insert fusion protein, which was labeled in an in vitro kinase reaction. The phosphopeptides common to both samples are labeled.

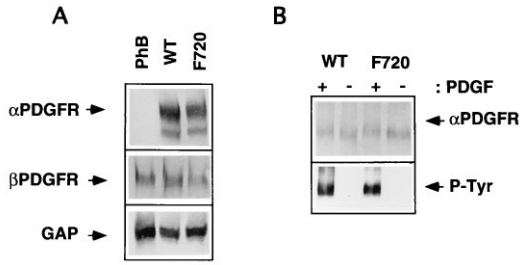

FIG. 3. Expression of the WT and the F720 αPDGFRs. Total cell lysates representing 4×10^4 cells were resolved on an SDS–7.5% polyacrylamide gel, transferred to Immobilon, and subjected to Western blot analysis using the antibodies indicated to the left of panel A and to the right of panel B. PhB is the parental cell line expressing only the endogenous βPDGFR, WT cells express the WT αPDGFR, and F720 cells express the phenylalanine mutant at position 720. (A) Lysates from unstimulated cells. (B) − and +, resting and PDGF-AA-stimulated cells, respectively.

Prior to testing whether mutating Y720 eliminated spot 8, we determined if the F720 mutant was capable of undergoing PDGF-stimulated receptor phosphorylation. Cells expressing the WT and the F720 αPDGFR were grown to confluence, starved overnight in DME plus 0.1% CS, and then left resting or stimulated with PDGF-AA. The cells were lysed, and the total cell lysates were analyzed by antiphosphotyrosine Western blot analysis (Fig. 3B). The resting cells contained no detectable tyrosine-phosphorylated αPDGFR, whereas exposure to PDGF strongly stimulated tyrosine phosphorylation of both the WT and the F720 αPDGFRs. Probing with an αPDGFR antibody demonstrated that similar amounts of receptor were present in all of the samples (Fig. 3B). These data demonstrate that the F720 receptor was fully competent to undergo PDGF-dependent tyrosine phosphorylation in vivo.

We then determined whether mutation of Y720 affected any of the spots in an αPDGFR phosphopeptide map. Cells expressing the WT or F720 αPDGFR were metabolically labeled with $^{32}P_i$ for 4 h and then either left resting or incubated for 10 min with PDGF-AA. Cells were lysed, and the αPDGFR was immunoprecipitated and analyzed by phosphopeptide mapping. As predicted by the experiments indicating that spot 8 is the phosphorylated Y720 peptide, mutating Y720 ablated spot 8 but did not have any effect on any of the other phosphopeptides (Fig. 1E). In addition, phosphopeptide maps of the WT and the F720 receptors labeled in vitro showed that spot 8 was missing from the F720 sample, whereas all of the other spots remained unchanged (data not shown).

In summary, several independent approaches identify Y720 as an in vitro and in vivo phosphorylation site in the kinase insert of the αPDGFR.

Receptor kinase activity of the F720 αPDGFR mutant. As shown in Fig. 3B, the F720 mutant was fully capable of undergoing PDGF-AA-dependent tyrosine phosphorylation in vivo. Additional experiments showed that PDGF-dependent tyrosine phosphorylation of the WT and the F720 mutant was indistinguishable over a time course of 5 to 45 min (data not shown). To further investigate the effect of the tyrosine to phenylalanine substitution at position 720 on the αPDGFR kinase activity, we compared the ability of the F720 and the WT receptors to autophosphorylate and to phosphorylate exogenous substrates in vitro. One of the exogenous substrates tested was a GST–PLCγ-1 fusion protein, encompassing the PLCγ-1 phosphorylation sites at positions Y771 and Y783. PDGFRs were immunoprecipitated from resting and PDGF-AA-stimulated cells and subjected to an in vitro kinase assay in the presence of 0.5 μg of GST–PLCγ-1 fusion protein. For both the WT and F720 receptors, stimulating cells with PDGF prior to immunoprecipitation resulted in an approximately three- to fourfold increase in the phosphorylation of the GST–PLCγ-1 fusion protein (Fig. 4A). Similar results were obtained with another exogenous substrate, a GST fusion protein including the C-terminal region of SHP-2 (data not shown). These data demonstrate that replacing Y720 with phenylalanine did not alter the kinase activity of the αPDGFR.

Y720 is required for binding of SHP-2. To test whether phosphorylation of Y720 is required for binding of the various receptor-associated proteins, confluent, quiescent PhB cells expressing the WT or the F720 αPDGFR were exposed to buffer or 50 ng of PDGF-AA per ml for 5 min at 37°C, and the receptor was immunoprecipitated with an αPDGFR antibody. The receptor-associated proteins were detected by phosphorylation in an in vitro kinase assay (Fig. 4A) or by Western blot analysis (Fig. 4B). The in vitro kinase assay showed that following stimulation with PDGF, proteins of 150, 120, and 85 kDa and a 64-kDa smear were readily detectable in the WT samples (Fig. 4A). The 85- and 64-kDa proteins have previ-

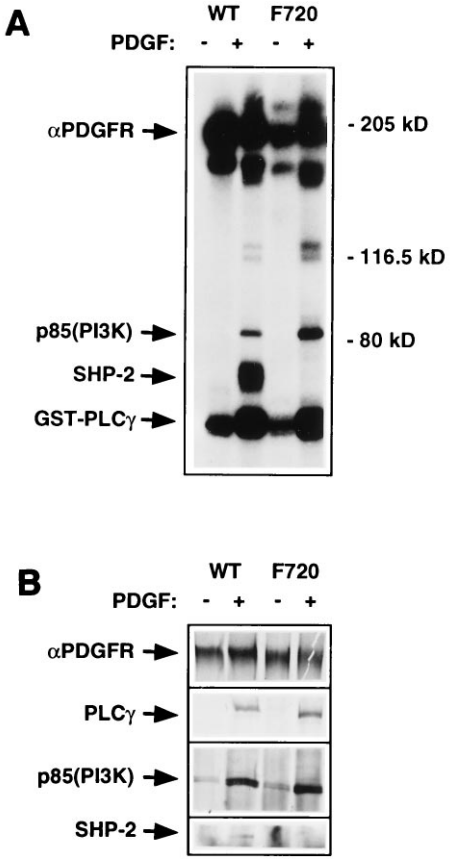

FIG. 4. The WT and the F720 αPDGFRs have similar in vitro kinase activities but bind to different sets of proteins. The WT and the F720 αPDGFRs were immunoprecipitated from resting (−) or PDGF-AA-stimulated (+) cells. (A) Immunoprecipitates representing 4×10^5 cells were subjected to an in vitro kinase assay in the presence of 0.5 μg of GST–PLCγ-1 fusion protein. The samples were resolved on an SDS–7.5% PAGE gel, and the resulting autoradiogram is presented. The arrows indicate the various PDGFR-associated proteins as well as the exogenous substrate (GST–PLCγ-1). Similar results were obtained in three independent experiments. (B) The αPDGFR immunoprecipitates were resolved on an SDS–7.5% polyacrylamide gel, transferred onto Immobilon, and immunoblotted with the indicated antisera. The associated proteins are indicated by the arrows.

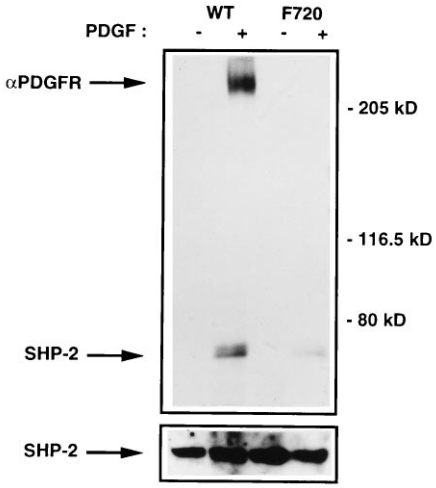

FIG. 5. Mutation of Y720 impairs SHP-2 tyrosine phosphorylation. SHP-2 was immunoprecipitated from resting (−) or PDGF-AA-stimulated (+) cells expressing the WT or the F720 mutant, and immunoprecipitates representing 4×10^6 cells were resolved by SDS-PAGE, transferred onto Immobilon, and immunoblotted with an antiphosphotyrosine antibody (upper panel) or with an SHP-2-specific antibody (lower panel). The labels on the left indicate the position of SHP-2 and the high-molecular-weight species, which is probably the αPDGFR. Similar results were obtained in two independent experiments.

ously been identified as p85 of PI3K and SHP-2, respectively (29, 60). The F720 receptor bound all of the same proteins, except that it failed to detectably associate with SHP-2. The presence of the exogenous substrate in the kinase assay whose results are shown in Fig. 4A had no effect on detection of receptor-associated proteins (data not shown). Western blot analysis indicated that following PDGF stimulation, the WT and F720 PDGFRs recruited comparable amounts of PLCγ-1, the p85 subunit of PI3K, and Src (Fig. 4B and data not shown). Consistent with the in vitro kinase assay, only the WT receptor associated with SHP-2 (Fig. 4B). These results are consistent with the prediction that the sequence surrounding Y720 is a possible binding site for the N-terminal SH2 domain of SHP-2 (53). We conclude that Y720 is required for binding of SHP-2 to the αPDGFR but not for the association of PLCγ, the p85 subunit of PI3K, or Src.

Tyrosine phosphorylation of SHP-2. Previous studies have shown that SHP-2 is tyrosine phosphorylated upon stimulation of cells with growth factors such as epidermal growth factor and PDGF and that it is constitutively tyrosine phosphorylated in v-*src*-transformed cells (18, 29, 32, 63). Binding of SHP-2 to the βPDGFR is not a strict requirement for PDGF-stimulated tyrosine phosphorylation of SHP-2. The F1009 βPDGFR mutant binds SHP-2 poorly but still initiates SHP-2 tyrosine phosphorylation to near WT levels (29). To determine whether binding of SHP-2 to the αPDGFR is a requirement for PDGF-AA-stimulated SHP-2 tyrosine phosphorylation, we compared PDGF-dependent SHP-2 tyrosine phosphorylation in cells expressing the WT and the F720 PDGFRs. Resting or PDGF-AA-stimulated cells were lysed, SHP-2 was immunoprecipitated, and the immunoprecipitates were analyzed by antiphosphotyrosine Western blotting (Fig. 5, upper panel). SHP-2 was not detectably tyrosine phosphorylated in resting cells expressing either the WT or the F720 receptor. In response to PDGF stimulation, SHP-2 was robustly tyrosine phosphorylated in cells expressing the WT receptor. These samples also contained a strongly tyrosine-phosphorylated high-molecular-weight species which was most probably the coimmunoprecipitating αPDGFR. In contrast, SHP-2 isolated from stimulated F720 cells was phosphorylated to less than 10% of the WT level. Coimmunoprecipitation of the αPDGFR was not detected, even though the F720 receptor is efficiently tyrosine phosphorylated in response to PDGF (Fig. 3B). A SHP-2 Western blot of these SHP-2 immunoprecipitates indicated that there was a similar amount of SHP-2 in each immunoprecipitates (Fig. 5, lower panel). These data strongly suggest that SHP-2 must bind to the αPDGFR to be efficiently tyrosine phosphorylated.

Y720 is also required for binding of Grb2. Grb2 binds to a number of tyrosine-phosphorylated proteins, including growth factor receptors as well as SHP-2. We performed the following experiments to determine whether Grb2 binds to the αPDGFR and whether mutating Y720 has any effect on Grb2 binding. The αPDGFR was immunoprecipitated from resting or PDGF-stimulated cells expressing the WT or the F720 PDGFR. As a positive control, the βPDGFR was immunoprecipitated from resting PDGF-stimulated parental cells. The amount of Grb2 coimmunoprecipitating with the various receptors was analyzed by Western blotting. The βPDGFR coimmunoprecipitated with a small fraction of the total Grb2 present in PhB cells (less than 0.05%), whereas no Grb2 was detected in the WT or F720 αPDGFR immunoprecipitates (Fig. 6A). We then employed a second assay to measure association of Grb2 with the αPDGFR. Grb2 was immunoprecipitated from resting or PDGF-stimulated cells, the samples were subjected to an in vitro kinase assay, the immune complex was denatured, and the samples were reimmunoprecipitated with an αPDGFR-specific antibody. By this assay we were able to detect the αPDGFR in Grb2 immunoprecipitates from PDGF-stimulated cells expressing the WT receptor (Fig. 6B). In contrast, only barely detectable levels of the F720 receptor were present in the Grb2 immunoprecipitates (Fig. 6B). These data show that both the α- and βPDGFRs detectably bind Grb2 and suggest that the βPDGFR associates with Grb2 more efficiently than the αPDGFR. Furthermore, efficient binding of Grb2 to the αPDGFR requires Y720.

SHP-2 binds directly to the αPDGFR, whereas Grb2 binds indirectly. Since Y720 was required for binding of both SHP-2 and Grb2, it raised the issue of how these two proteins interact with the αPDGFR. For the βPDGFR, Grb2 associates with the receptor directly, once Y716 is phosphorylated, or indirectly, where Grb2 binds the receptor via tyrosine-phosphorylated SHP-2 (1, 7, 34). In contrast, SHP-2 appears to associate with the βPDGFR directly (7, 29, 34). The mechanism by which either Grb2 or SHP-2 associates with the αPDGFR was not known, and we decided to further investigate this question. To test whether SHP-2 binds directly to the receptor, we looked at the ability of unphosphorylated SHP-2 to associate with the phosphorylated receptor in an in vitro binding assay. The WT or F720 receptor was immunoprecipitated from unstimulated cells, phosphorylated in vitro with unlabeled ATP, and then incubated with lysates of resting PhB cells, where SHP-2 is not detectably tyrosine phosphorylated (Fig. 5 and data not shown). The immunoprecipitates were washed and subjected to a standard in vitro kinase assay. Unphosphorylated receptors were used as a negative control. Figure 7A shows that the phosphorylated WT and F720 receptors were both able to bind numerous proteins, including a doublet at around 120 kDa and a prominent 85-kDa species. Only the WT receptor could associate with the 64-kDa smear, SHP-2. The unphosphorylated WT and F720 receptors failed to bind any of these proteins. These data demonstrate that SHP-2 does not need to be tyrosine phosphorylated in order to bind to the phosphorylated αPDGFR and are consistent with the idea that SHP-2 binds to the αPDGFR directly.

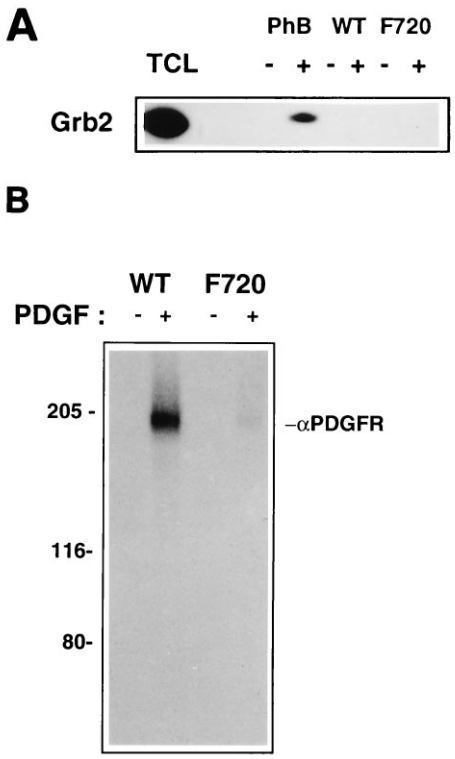

FIG. 6. Interaction of Grb2 with the αPDGFR. (A) The WT and the F720 αPDGFRs were immunoprecipitated from resting (−) or PDGF-AA-stimulated (+) cells. Immunoprecipitates representing 4×10^5 cells were resolved on an SDS–7.5% polyacrylamide gel, transferred onto Immobilon, and immunoblotted with an Grb2-specific antibody. In the lanes labeled "PhB," the βPDGFR was immunoprecipitated from resting (−) or PDGF-BB-stimulated (+) parental PhB cells. The TCL lane is 1% of a total cell lysate from unstimulated PhB cells. (B) Grb2 was immunoprecipitated from resting (−) or PDGF-AA-stimulated (+) Ph cells expressing the WT or F720 αPDGFR. The Grb2 immunoprecipitates were first subjected to an in vitro kinase assay, the immune complex was disrupted by boiling the samples in the presence of SDS and dithiothreitol, and then the samples were reimmunoprecipitated with an αPDGFR-specific antibody. The samples were resolved by SDS-PAGE, and the resulting gel was exposed to film. Similar results were obtained in two independent experiments.

We next examined whether Grb2 binds to the αPDGFR directly or indirectly. We were consistently unable to demonstrate that Grb2 binds to the αPDGFR in an in vitro binding assay, as described above (data not shown), which could mean that Grb2 does not associate with the αPDGFR directly. These findings encouraged us to consider the possibility that Grb2 binds to the αPDGFR indirectly, perhaps via SHP-2, as it does with the βPDGFR. Note also that the sequence downstream of Y720 (Y^{720}VILSF) is not an optimal binding sequence for the Grb2 SH2 domain (pY[Q/V/N]N[Y/Q/F]), whereas Y580 in the C terminus of SHP-2 is an optimal and demonstrated Grb2 binding site (Y^{580}ENVGL) (7, 34, 53, 54). Consequently, we tested if SHP-2 is tyrosine phosphorylated at position 580 and if it associates with Grb2 in response to PDGF-AA stimulation.

SHP-2 was immunoprecipitated from in vivo-labeled resting or PDGF-AA-stimulated cells expressing the WT αPDGFR and analyzed by phosphopeptide mapping. PDGF stimulated the appearance of numerous spots (Fig. 7B). To determine which if any of these PDGF-inducible phosphopeptides was the Y580 peptide, we generated a radiolabeled Y580 phosphopeptide standard as follows. A GST fusion protein which included a portion of SHP-2's C terminus that contained only a single tyrosine, Y580, was radiolabeled in a standard in vitro kinase assay. GST itself was poorly phosphorylated under these conditions (data not shown). Phosphopeptide mapping of this fusion protein revealed that the major species corresponded to spot 1 of the SHP-2 map (data not shown). Thus, SHP-2 is phosphorylated at Y580 in PDGF-AA stimulated

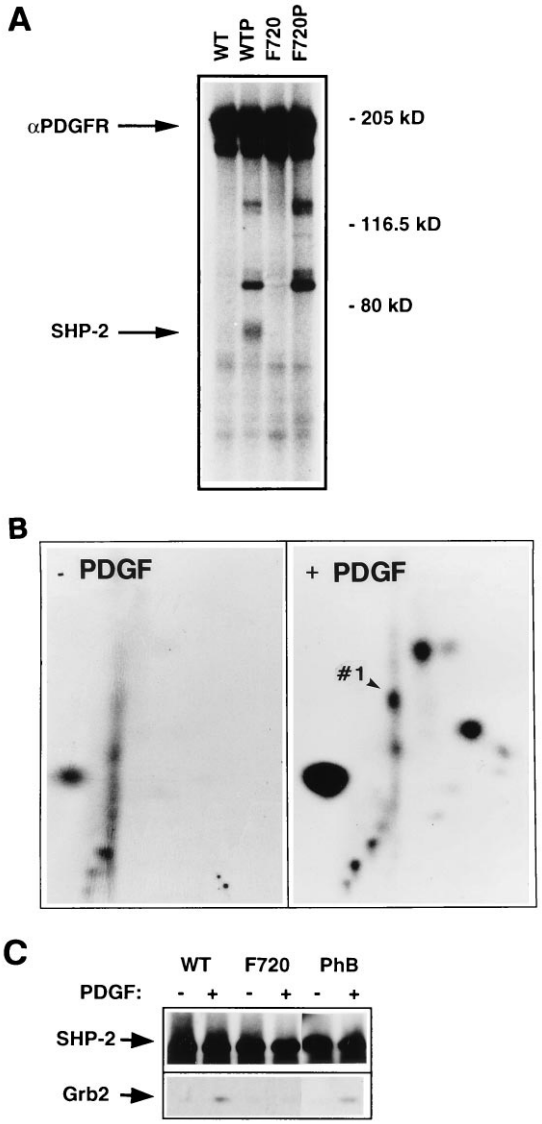

FIG. 7. Grb2 binds indirectly to the αPDGFR via SHP-2. (A) The αPDGFR was immunoprecipitated from approximately 10^6 resting cells expressing WT and F720 αPDGFRs, phosphorylated with cold ATP in vitro (WTP and F720P), and incubated with lysate from 2×10^6 unstimulated parental PhB cells for 2 h on ice. The samples were washed, subjected to an in vitro kinase assay using [γ-^{32}P]ATP, and resolved on an SDS–7.5% polyacrylamide gel, and the resulting autoradiogram is presented. The WT and F720 lanes show results of the control experiments using unphosphorylated receptor immunoprecipitates. Similar results were obtained in two independent experiments. (B) Cells expressing the WT αPDGFR were labeled with $^{32}P_i$, left resting (left panel) or stimulated with PDGF-AA (right panel), and lysed, and SHP-2 was immunoprecipitated. SHP-2 isolated from 6×10^6 cells was analyzed by tryptic and thermolytic phosphopeptide mapping as described in Material and Methods. The arrow points to the Y580 phosphopeptide. (C) SHP-2 was immunoprecipitated from resting (−) or PDGF-AA-stimulated (+) cells expressing the WT or F720 αPDGFR. The immunoprecipitates (representing 4×10^6 cells) were resolved by SDS-PAGE, transferred onto Immobilon, and analyzed by SHP-2 (upper panel) or Grb2 (lower panel) Western blotting. Lanes PhB, parental cells stimulated with PDGF-BB instead of PDGF-AA.

cells, and this observation indicates that SHP-2 could be forming a stable complex with Grb2.

To further test this possibility we immunoprecipitated SHP-2 from resting or PDGF-stimulated cells and checked for Grb2 by subjecting the immunoprecipitates to anti-Grb2 Western blot analysis. Figure 7C shows that Grb2 coimmunoprecipitates with SHP-2 from PDGF-stimulated WT receptor-expressing cells but not from the F720 cells. A likely explanation for the failure of the Grb2–SHP-2 complex to stably associate following activation of the F720 receptor is that SHP-2 is poorly phosphorylated in the F720-expressing cells (Fig. 5). SHP-2 was also detected in Grb2 immunoprecipitates from PDGF-AA-stimulated cells expressing the WT receptor (data not shown). The SHP-2–Grb2 complex probably does not include the αPDGFR, since Grb2 was not detected in αPDGFR immunoprecipitates (Fig. 6A). Instead, phosphorylation of SHP-2 at Y580 may enable Grb2 to bind directly to SHP-2. Activation of the βPDGFR in the parental Ph cells also initiated formation of a complex between SHP-2 and Grb2, and the amounts of this complex were comparable when either of the PDGFRs was activated (Fig. 7C). In summary, these data demonstrate that activation of the αPDGFR leads to phosphorylation of SHP-2 at Y580, which appears to trigger the formation of a complex between Grb2 and SHP-2. In addition, SHP-2 binds the activated αPDGFR directly, whereas Grb2 binds indirectly, via SHP-2.

Activation of Ras. If binding of Grb2 or SHP-2 to the αPDGFR is required for activation of Ras, then the F720 mutant, which does not bind detectable levels of either of these proteins, should be unable to drive accumulation of GTP-bound Ras. To test this possibility, we compared the ability of the WT and F720 receptors to activate Ras. In addition, we measured Ras activation in response to PDGF-BB, which engages both the introduced αPDGFR and the endogenous βPDGFR. Confluent, quiescent cells expressing the WT and the F720 αPDGFRs were labeled with $^{32}P_i$ and exposed to buffer, 30 ng of PDGF-BB per ml, or 50 ng of PDGF-AA per ml for 5 min at 37°C; then, the cells were lysed, Ras was immunoprecipitated, and the Ras-associated nucleotides were resolved by thin-layer chromatography. Engaging only the αPDGFR (with PDGF-AA) resulted in activation of Ras, and the WT and the F720 αPDGFRs were equally able to induce this response (Fig. 8). A kinetic analysis of Ras activation demonstrated that there was no consistent difference between the WT and the F720 receptors in their ability to stimulate accumulation of GTP-Ras at 3, 5, 7.5, 10, and 15 min following stimulation with PDGF-AA (data not shown). PDGF-BB, which engaged both PDGFRs, did not cause a greater activation of Ras (Fig. 8), and stimulation of only the βPDGFR in the parental cells induced a comparable response (data not shown). These data indicate that both the α- and βPDGFRs stimulate maximal Ras activation and that for the αPDGFR, this event does not require detectable binding of Grb2 or SHP-2. Furthermore, either Grb2 and SHP-2 do not contribute to PDGF-AA-mediated Ras activation or the αPDGFR engages multiple pathways leading to stimulation of Ras.

Biological responses. We also investigated the importance of Grb2 and SHP-2 in αPDGFR-mediated mitogenic signal relay. In order to compare the ability of the WT and the F720 αPDGFRs to trigger DNA synthesis, receptor-expressing cells were arrested by serum deprivation and stimulated with increasing concentrations of PDGF-AA, and then thymidine incorporation was measured. PDGF stimulated comparable dose responses in cells expressing either the WT and F720 αPDGFRs (Fig. 9A). In addition, the maximal response to PDGF-AA for both cell types was essentially identical to the response to PDGF-BB (Fig. 9A and data not shown), indicating that the αPDGFR was able to drive a maximal DNA synthesis response. These studies indicate that the α- and βPDGFRs initiate similar DNA synthesis responses and that interaction of the αPDGFR with SHP-2 and Grb2 is not required for this event.

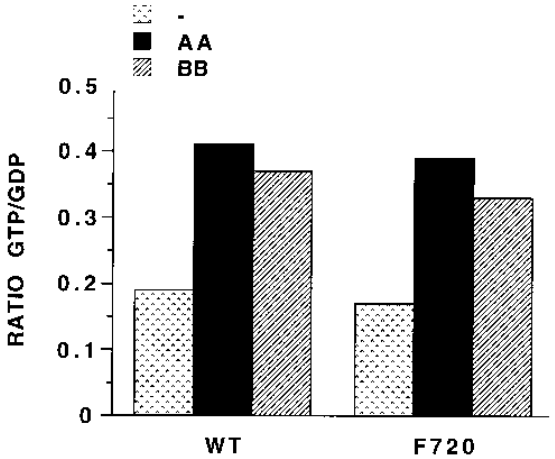

FIG. 8. Activation of Ras by the WT and F720 αPDGFRs. Confluent, quiescent PhB cells expressing either the WT or the F720 αPDGFR were labeled with $^{32}P_i$ and either left unstimulated or stimulated with 30 ng of PDGF-BB or 50 ng of PDGF-AA per ml for 5 min at 37°C; the cells were lysed, and Ras was immunoprecipitated by using the Y13-259 antibody. The Ras-associated nucleotides were resolved on a polyethyleneimine cellulose plate, and the radioactive GDP and the GTP were quantitated with a Molecular Dynamics PhosphorImager. The data are expressed as the ratio of radioactivity in GTP/to that in GDP and are representative of a total of four independent experiments.

We also compared the abilities of the WT and the F720 αPDGFR-expressing cells to grow in soft agar, which is a measure of the proliferative signal. In the absence of added growth factor, there was no detectable growth for any of the cell lines (Fig. 9B). In the presence of PDGF-AA, the WT and F720-expressing cells formed colonies of similar sizes and quantities, whereas parental or empty vector-containing cells did not respond (Fig. 9B and data not shown). These results indicate that the αPDGFR is able to promote transition through the cell cycle and growth in soft agar without associating with Grb2 or SHP-2. Consequently, either these receptor-associated proteins are not required for the mitogenic response or the αPDGFR drives cell growth via several distinct signaling cascades.

DISCUSSION

Exposing cells to PDGF-AA leads to phosphorylation of the αPDGFR at numerous tyrosine residues, and we identify one of these sites as Y720. Phosphorylation of Y720 is required for recruitment of SHP-2 and Grb2 to the αPDGFR. In addition, SHP-2 binds to the αPDGFR directly, whereas Grb2 associates with the αPDGFR indirectly, via tyrosine-phosphorylated SHP-2. Furthermore, the αPDGFR is fully capable of activating Ras and promoting cellular proliferation without recruiting SHP-2 or Grb2. Finally, identification of the SHP-2 binding site in distinct locations in the α- and βPDGFRs strengthens the idea that these two closely related PDGFRs are nevertheless quite different.

Activation of the αPDGFR leads to an accumulation of GTP-bound Ras and stable association of Grb2 with the αPDGFR. Since Grb2 has been shown to contribute to Ras activation by receptor tyrosine kinases (10, 42, 48), we were

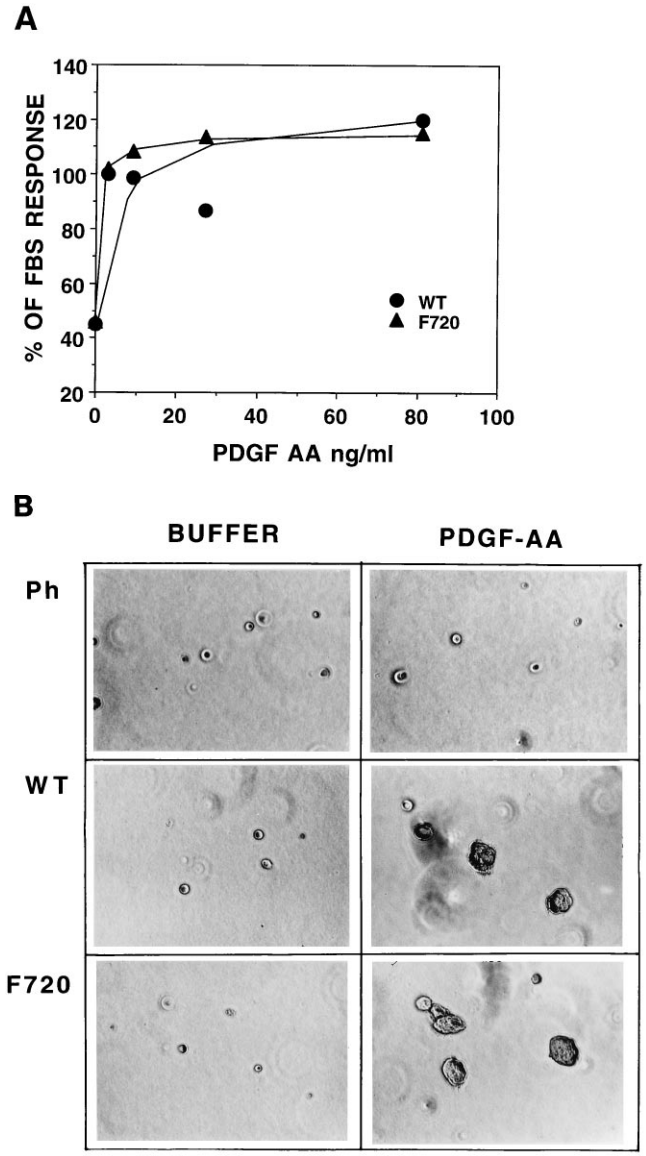

FIG. 9. Mitogenic signaling by the F720 αPDGFR. (A) Cells were grown to confluence, arrested by serum deprivation, and then stimulated with the indicated concentration of PDGF. After an 18-h incubation the cells were pulsed with [^{3}H]thymidine for 4 h and harvested, and the incorporated radioactivity was quantitated. The data are expressed as percentage of the response to 10% fetal bovine serum, which routinely initiated a two- to threefold stimulation. The standard error of the mean was less than 10%, and four independent experiments gave similar results. (B) Soft-agar assay. Cells were plated in DME medium containing 0.45% agarose and 5% CS, incubated at 37°C for 10 days, and then photographed. The photographs are of representative areas of the dish, and three independent experiments gave similar results.

surprised that preventing detectable binding of Grb2 did not at least diminish stimulation of Ras (Fig. 8). It is possible that the small amount of Grb2 that still binds to the F720 αPDGFR is sufficient to activate Ras. Alternatively, the αPDGFR engages the Ras pathway without the help of Grb2. This observation is reminiscent of the βPDGFR signaling cascade, in which we have also not been able to tightly correlate Grb2 binding to the βPDGFR with Ras activation (60). Our attempts to identify the pathways by which the αPDGFR activates Ras have focused on Shc; however, Shc was not detectably phosphorylated, nor did it associate with other tyrosine-phosphorylated proteins when immunoprecipitated from resting or PDGF AA-stimulated Ph cells expressing the WT αPDGFR (unpublished observations). While it is possible that Grb2 does bind to the F720 receptor at a very low level and that this is sufficient to activate Ras, it seems more likely that the αPDGFR stimulates Ras by some alternative pathway. Furthermore, the role of Grb2 in αPDGFR signaling may be related to Grb2's ability to act as an adapter for proteins other than those involved in Ras activation or sending a mitogenic signal.

A key question regarding the role of SHP-2 in αPDGFR signaling is the identification of relevant SHP-2 substrates in this signal transduction cascade. In several signaling pathways downstream of receptor tyrosine kinases, SHP-2 appears to participate in events leading to activation of mitogen-activated protein kinases (6, 35, 58, 64, 65). An apparently distinct SHP-2 function has been identified following activation of the βPDGFR (31). SHP-2 dephosphorylates the βPDGFR at a select group of tyrosine residues, primarily Y751, which contributes to binding of PI3K, as well as Y771, which is required for binding of RasGAP. Unlike the βPDGFR, binding of SHP-2 to the αPDGFR does not appear to affect the phosphorylation state of the αPDGFR, as indicated by the identical time course of αPDGFR dephosphorylation for the WT and F720 mutants (unpublished observations). Thus, either SHP-2 acts on a small set of αPDGFR phosphorylation sites and the loss of these sites is not detected when the bulk αPDGFR phosphorylation state is examined, or SHP-2 does not dephosphorylate the αPDGFR. An alternate role for SHP-2 in αPDGFR signaling is that SHP-2 acts to dephosphorylate certain αPDGFR-associated proteins. Src is an obvious SHP-2 substrate, since activation of the αPDGFR leads to increased Src activity (19a) and dephosphorylation of the negative regulatory site in Src's C terminus activates Src kinase activity (11, 12). However, PDGF-AA-mediated activation of Src was indistinguishable in cells expressing the WT and those expressing the F720 mutant (data not shown). Similar experiments examining PLCγ tyrosine phosphorylation by the WT and F720 αPDGFRs indicated that SHP-2 did not regulate the phosphorylation state of PLCγ (data not shown). Thus, SHP-2 does not appear to dephosphorylate the αPDGFR itself or some of the αPDGFR-associated proteins. Additional studies are under way to further investigate the role of SHP-2 in αPDGFR signal relay and to identify relevant substrates. This information is expected to shed light on the more general question of the significance of binding the same signaling molecule at different locations on the two PDGFRs.

Which, if any, of the signal relay enzymes that associate with the αPDGFR are the intracellular effectors of the αPDGFR? While it is problematic to compare the signaling properties of receptors expressed in different cell lines, studies to date have shown that individually removing PI3K, SHP-2, and PLCγ does not diminish the ability of the αPDGFR to initiate DNA synthesis (15, 68–70). This sharply contrasts with the observation that removing the binding sites for PI3K, SHP-2, or PLCγ at least partially affect the βPDGFR's ability to transduce a signal to initiate DNA synthesis in most of the cell types tested (26). Consequently, signaling by the αPDGFR and that by the βPDGFR appear to be distinct processes, despite the recruitment of a similar set of SH2 domain signal relay molecules. The observation that binding of PLCγ is not required for initiation of DNA synthesis by the fibroblast growth factor receptor (36, 43) while it is required for the βPDGFR in certain cell types (59, 60) suggests that all receptors do not have the same signal relay requirements for initiating a mitogenic response. Furthermore, mutating the epidermal growth factor receptor so that it can no longer stably associate with

SH2 domain-containing proteins does not diminish the ability of this receptor to signal (33, 52). Clearly, receptor tyrosine kinases are capable of triggering numerous signaling cascades. Ongoing efforts with the αPDGFR are focused on determining the relative contribution of each of the αPDGFR-associated proteins to the initiation of DNA synthesis.

The data presented herein reveal additional differences between the closely related α- and βPDGFR receptor tyrosine kinases. Firstly, the only detectable mechanism by which Grb2 associates with the αPDGFR is an indirect route, via SHP-2, whereas Grb2 binds to the βPDGFR by both direct and indirect mechanisms. Secondly, robust tyrosine phosphorylation of SHP-2 requires stable association with the αPDGFR, but for the βPDGFR these two events are less tightly correlated. Finally, SHP-2 binds to the kinase insert of the αPDGFR, whereas it binds to the carboxy-terminal tail of the βPDGFR. Further elucidation of the αPDGFR signaling cascades and comparison with the βPDGFR will provide a solid foundation for understanding how receptor tyrosine kinases initiate and regulate intracellular signaling pathways.

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