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The GTPase-Activating Protein of Ras Suppresses Platelet-Derived Growth Factor β Receptor Signaling by Silencing Phospholipase C-γ1

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The β receptor for platelet-derived growth factor (βPDGFR) is activated by binding of PDGF and undergoes phosphorylation at multiple tyrosine residues. The tyrosine-phosphorylated receptor associates with numerous SH2-domain-containing proteins which include phospholipase C-γ1 (PLCγ), the GTPase-activating protein of Ras (GAP), the p85 subunit of phosphatidylinositol 3 kinase (PI3K), the phosphotyrosine phosphatase Syp, and several other proteins. Our previous studies indicated that PI3K and PLCγ were required for relay of the mitogenic signal of βPDGFR, whereas GAP and Syp did not appear to be required for this response. In this study, we further investigated the role of GAP and Syp in mitogenic signaling by βPDGFR. Focusing on the PLCγ-dependent branch of βPDGFR signaling, we constructed a series of mutant βPDGFRs that contained the binding sites for pairs of the receptor-associated proteins: PLCγ and PI3K, PLCγ and GAP, or PLCγ and Syp. Characterization of these mutants showed that while all receptors were catalytically active and bound similar amounts of PLCγ, they differed dramatically in their ability to initiate DNA synthesis. This signaling deficiency related to an inability to efficiently tyrosine phosphorylate and activate PLCγ. Surprisingly, the crippled receptor was the one that recruited PLCγ and GAP. Thus, GAP functions to suppress signal relay by the βPDGFR, and it does so by silencing PLCγ. These findings demonstrate that the biological response to PDGF depends not only on the ability of the βPDGFR to recruit signal relay enzymes but also on the blend of these receptor-associated proteins.

For the β receptor of the platelet-derived growth factor (βPDGFR), ligand binding activates its intrinsic kinase activity and leads to phosphorylation at multiple tyrosine residues. The phosphorylated receptor associates with a set of SH2 domain-containing proteins that includes phospholipase C-γ1 (PLCγ), the GTPase-activating protein of Ras (GAP), the regulatory subunit of phosphatidylinositol 3 kinase (PI3K), the phosphotyrosine phosphatase Syp (also called SH-PTP2, PTP-1D, SH-PTP3, PTP2C, and PTP-SH2β), three members of the Src family (Src, Yes, and Fyn), Nck, Shc, Grb2, and several as yet unidentified proteins (1, 30, 82). These proteins associate with the phosphorylated receptor by a variety of mechanisms. Some proteins have specific binding sites on the receptor and associate only when the receptor is phosphorylated at these sites. For instance, binding of PLCγ, GAP, Syp, and PI3K requires phosphorylation of the receptor at tyrosines 1021, 771, 1009, and 740/751, respectively (30). Proteins such as Shc do not have a strict phosphorylation site requirement (82), and their binding to the βPDGFR is reminiscent of how SH2-domain containing proteins associate with the epidermal growth factor receptor (68). Grb2 appears to associate with the receptor via multiple mechanisms, either by binding to Syp, which then complexes with the phosphorylated receptor (41), or by binding directly to the receptor in response to phosphorylation of tyrosine 716 (1).

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At least some of the receptor-associated proteins are key players in intracellular signal transduction cascades. For instance, PLCγ is a phosphatidylinositol-specific phosphodiesterase that is activated by tyrosine phosphorylation in response to engagement of a wide variety of cell surface receptors (reviewed in reference 60). The possibility that PLCγ plays a direct role in mitogenic signal transduction is also supported by the observation that microinjection of purified PLCγ initiates a DNA synthesis response (67). Thus, PLCγ is involved in signal relay by a variety of cell surface receptors and, in certain situations, couples to mitogenic signaling pathways.

One of the known functions of GAP is to regulate the steady-state level of activated Ras. Ras cycles between active (GTP-bound) and inactive (GDP-bound) forms, and the balance between these two states is achieved by the collective action of GAP, nucleotide exchange factors, and nucleotide-releasing factors (3). Numerous studies have shown that Ras plays a central role in enabling a cell to respond to mitogenic signals, and consequently there has been great interest in understanding the details of how Ras is regulated. In phorbol ester-stimulated T cells, nucleotide exchange activity is invariant, while cellular GAP activity decreases, suggesting that Ras activation involves regulation of GAP activity in T cells (13). In contrast, GAP activity does not change in a growth factor-stimulated fibroblast, but both activation of nucleotide exchange activity and the redistribution of certain nucleotide exchange factors (such as SOS) are the key events associated with accumulation of active Ras (6, 53, 83). Thus, while changes in GAP activity appear directly responsible for the increase in active Ras in certain cell types, this does not seem to be the primary function of GAP in a growth factor-stimulated fibroblast.

Perhaps the most informative studies regarding the role of

GAP in mammalian cells have involved overexpression of the N- or C-terminal portion of GAP and have revealed that the two halves of GAP mediate distinct biological functions. The N-terminal half of GAP consists of approximately 500 amino acids that encodes two SH2, one SH3, and one PH (46) domain, whereas the C-terminal portion contains the catalytic domain responsible for activating the GTPase activity of Ras (44). The C-terminal portion is able to suppress the growth and transformation of cells (4, 10, 11, 26), whereas the N-terminal half inhibits K^+ channel inactivation (45), enhances c-*fos* expression (48), inhibits transformation by G protein-coupled receptors (81), disrupts the cytoskeleton, and diminishes cell adhesion (47). A comparison of the response of cells overexpressing full-length GAP or just the N or C termini indicated that full-length GAP often failed to mediate the responses observed with the truncated forms. These findings have led to the theory that the two halves of GAP negatively regulate each other's function and that binding of Ras unfolds GAP, enabling the two domains to mediate their separate biological effects (45). Thus, it appears that GAP can regulate a number of biological processes and that there are several distinct functional domains within the GAP protein.

Since many of the receptor-associated proteins are members of signal relay cascades, it seems likely that they are the intracellular effector proteins responsible for relaying the receptor's biological directives. A variety of approaches have been used to test this hypothesis. Microinjection of DNA encoding kinase-inactive Src or Fyn or of antibodies that inhibit PI3K activity blocked PDGF-dependent entry into the cell cycle, indicating that these enzymes play an important role in PDGF-dependent mitogenic signaling (61, 73). A second approach has been to study the signaling capacity of βPDGFR phosphorylation site mutants which fail to associate with one of the receptor-associated proteins (15, 36, 51, 62, 74, 75). This approach has also indicated an important role for PI3K in βPDGFR signaling, but a consensus role for the other receptor-associated proteins has not emerged. While PI3K may be the only receptor-associated protein important in PDGF-mediated mitogenesis, an alternate explanation is that the βPDGFR is sending multiple signals, so that the biological consequence of losing one of the receptor-associated proteins is minimized by the presence of redundant signaling cascades. The possibility that the βPDGFR initiates multiple signaling pathways has been addressed by studying a mutant receptor (F5) unable to bind many of the receptor-associated proteins (PI3K, PLCγ, GAP, and Syp) (75). While this mutant was unable to drive PDGF-dependent DNA synthesis, restoration of either the PI3K- or PLCγ-binding site restored the majority of the DNA synthesis response. In contrast, Syp or GAP binding did not rescue the signaling of the F5 PDGFR. In summary, multiple approaches have been used to investigate the role of the receptor-associated proteins in βPDGFR mitogenic signaling, and it appears that PI3K, PLCγ, and Src family members are important for PDGF-dependent mitogenic signaling. Despite the large efforts by multiple groups, a largely unanswered question is the contribution of GAP and Syp to βPDGFR signal relay. The observation that removing the βPDGFR's GAP-binding site improves the PDGF-dependent chemotactic response (39) raises the possibility that GAP has a negative role in signaling by the βPDGFR.

To study the role of GAP and Syp in mitogenic signaling by the βPDGFR, we constructed a series of mutant βPDGFRs that contained the binding sites for a pair of the receptor-associated proteins: PLCγ and PI3K, PLCγ and GAP, or PLCγ and Syp. Characterization of these mutants revealed that while all receptors were catalytically active and bound similar amounts of PLCγ, they differed dramatically in their ability to initiate DNA synthesis. This signaling deficiency related to an inability to efficiently tyrosine phosphorylate and activate PLCγ, and the problem receptor was the one that associated with PLCγ and GAP. Consequently, these studies strongly suggest that GAP acts to prevent signal relay by the βPDGFR and that it does so by silencing PLCγ. Furthermore, these studies reveal that the biological response to PDGF depends not only on the ability of the βPDGFR to recruit signal relay enzymes but also on the precise blend of these receptor-associated proteins.

MATERIALS AND METHODS

Cell lines. The HepG2 cells were maintained as previously described (75). We have recently found that there is a small amount of PDGFα receptor (αPDGFR) expressed in the HepG2 cells which can be detected in an in vitro kinase assay of an αPDGFR immunoprecipitate. Attempts to quantitate the level of expression have been unsuccessful, since the αPDGFR was not detected in αPDGFR immunoprecipitates isolated from ^{35}S-labeled cells or by Western immunoblotting of these immunoprecipitates (data not shown). Importantly, this barely detectable amount of αPDGFR does not appear to contribute to any of the responses measured (see Fig. 3, 4, and 7). A431 cells, a human epithelioid cell line, were obtained from Lynn Heasley, University of Colorado Health Science Center, Denver, and were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 50 U of penicillin G per ml, 50 μg of streptomycin per ml, and 5% fetal bovine serum (FBS). The A431 cells contain no detectable αPDGFR or βPDGFR (data not shown), consistent with previous observations (23).

Construction of βPDGFR mutants. Construction of the F5, Y1021, and Y21/09 (called F740/F751/F771 in reference 36) mutants has been previously described (36, 75). To make the Y21/40/51 and Y21/71 receptors, the 2.8-kb *Eco*RI-*Bam*HI fragment from F771 and F740/F751 receptors (36), respectively, were subcloned into Y1021/pVZ (33). The resulting receptor constructs were subcloned (as a 4.2-kb *Eco*RI-*Sal*I fragment) into the pLXSN retroviral vector (50) and introduced into the Ψ2 and PA317 virus-producing cell lines (33), and the resulting virus was used to infect HepG2 or A431 cells. Mass populations of cells were selected on the basis of their ability to grow in the presence of G418 at 1 mg/ml (active concentration), and after the initial round of selection, the cells were routinely maintained in the medium described above supplemented with 0.5 mg of G418 per ml.

Characterization of the βPDGFR mutants. Confluent, quiescent cultures of cells expressing the various βPDGFR mutants were left resting or stimulated with 50 ng of PDGF-BB per ml for 5 min at 37°C, the cells were lysed in EB (32), and the receptor was immunoprecipitated with an anti-βPDGFR antibody (30A [75]) exactly as described previously (36). Immunoprecipitates representing approximately 4.2×10^6 cells were resolved on a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and transferred to Immobilon, and different regions of the blot were analyzed by Western blot analysis with specific antisera, as previously described (75).

To assay for Syp association with the receptor, cells were left resting or stimulated with PDGF-BB and then lysed in EB, anti-Syp antibody (35) was added to the lysate, and the resulting immune complexes were collected on a suspension of formalin-fixed *Staphylococcus aureus* and washed as described previously (36). Immunoprecipitates representing approximately 1.8×10^6 cells were subjected to a standard in vitro kinase assay [10-min incubation at 30°C in 20 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES; pH 7.0), 10 mM $MnCl_2$, 20 μg of aprotinin per ml], the proteins were resolved by SDS-PAGE (7.5% polyacrylamide), and gel was alkali treated (31), and the radiolabeled proteins were detected by autoradiography.

To compare the ability of the different receptor mutants to phosphorylate an exogenous substrate, the βPDGFR was immunoprecipitated with PR7212 (a mouse monoclonal antibody recognizing an extracellular epitope of the βPDGFR [21]), and immunoprecipitates from approximately 7×10^5 cells were subjected to the standard in vitro kinase assay which was modified by the inclusion of 0.5 μg of a purified glutathione *S*-transferase (GST-PLC) fusion protein. The fusion protein included amino acids 550 to 850 of rat PLCγ. The proteins were resolved by SDS-PAGE(7.5% polyacrylamide), and the radiolabeled proteins were detected by autoradiography.

To examine PDGF-dependent phosphorylation of GAP and pp120, HepG2 cells expressing the receptor mutants were grown to confluence in DMEM–10% FBS, starved for 24 to 48 h in serum-free DMEM, left resting or stimulated for 7 min with 50 ng of PDGF-BB per ml (for GAP immunoprecipitates) or 5 and 100 ng of PDGF-BB (for pp120 immunoprecipitates) per ml, lysed in EB, and immunoprecipitated with the previously described GAP antibody (74) or with a commercially available pp120 monoclonal antibody (no. P17920; Transduction Labs). Immunoprecipitation was carried out exactly as described above, except that 1 μg of rabbit anti-mouse secondary antibody was used in the pp120 samples. Immunoprecipitates representing 1.2×10^6 cells were resolved by SDS

PAGE (7.5% polyacrylamide) and subjected to antiphosphotyrosine Western blot analysis as previously described (74).

DNA synthesis. The DNA synthesis response of HepG2 cells expressing the various mutant receptors was tested at different concentrations of PDGF exactly as previously described (75).

Activation of PLCγ. Production of inositol phosphates in HepG2 and A431 cells expressing the various PDGFR mutants was determined exactly as described previously (74), except that 70 ng of PDGF-BB per ml was used to stimulate the A431 cells.

To determine the PLC activity present in antiphosphotyrosine immunoprecipitates, confluent quiescent cultures of receptor-expressing HepG2 cells were either left quiescent or stimulated for 5 min with 100 ng of PDGF-BB per ml. The cells were solubilized in EB, and immunoprecipitates were prepared by incubating the cellular lysates with 1G2-coupled Sepharose beads (32) at 4°C for 45 min. Immunoprecipitates were washed twice with EB and twice with reaction buffer [50 mM sodium phosphate (pH 6.8), 100 mM KCl, 1 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), and 0.3% (wt/vol) sodium cholate]. Bound proteins were eluted from the antibody matrix with reaction buffer containing 10 mM phenyl phosphate, one-fourth of the eluate (representing 5×10^5 cells) was then removed and diluted to 72 μl with reaction buffer, and the final concentration of $[Ca^{2+}]$ was adjusted to 1 mM with $CaCl_2$. PLCγ reactions were performed at 37°C by addition of 3 μl of substrate solution, which was prepared by sonication of 1 mM phosphatidyl-[2-^{3}H]inositol 4,5-bisphosphate ([2-^{3}H]PI-4,5-P_2; specific activity, 17 Ci/mmol; NEN) in aqueous 5% (wt/vol) sodium cholate. The reactions were terminated after 15 min by adding 270 μl of chloroform-methanol-concentrated HCl (1:2:0.2); this was followed by the addition of 90 μl of chloroform and 90 μl of 1 M HCl containing 5 mM EGTA. The samples were vortexed vigorously and centrifuged to separate the phases. Radioactivity in the upper (aqueous) phase was quantitated by liquid scintillation counting. The remainder of each phenyl phosphate eluate (representing 1.5×10^6 cells) was separated by SDS-PAGE (7.5% polyacrylamide), transferred to Immobilon, and subjected to Western blot analysis with the mixture of PLCγ-specific antibodies (no. 05-163; UBI).

PLCγ tyrosine phosphorylation. The extent of PLCγ tyrosine phosphorylation in HepG2 cells expressing the various βPDGFR mutants was compared as follows. Cells were grown to confluence in DMEM–10% FBS, then switched to DMEM–0.1% calf serum for 24 to 48 h. The cells were then either left resting or stimulated with 50 ng of PDGF-BB per ml for 5 min at 37°C and lysed in EB, and PLCγ was immunoprecipitated from the lysates with a mixture of monoclonal antibodies as described above. Immunoprecipitates representing 4×10^6 cells were resolved by SDS-PAGE (7.5% polyacrylamide), the proteins were transferred to Immobilon, and the appropriate portion of the blot was probed with the 30A anti-βPDGFR antibody or a mixture of antiphosphotyrosine antibodies (PY20-4G10; 1:1). A portion of the same samples (representing 1.2×10^6 cells) was also subjected to a PLCγ Western blot to determine whether the same amount of PLCγ was present in all of the samples.

For the kinetic experiments, all of the procedures were the same as described above, except that the cells were cooled to 4°C and stimulated with 80 ng of PDGF-BB per ml for the amount of time indicated in Fig. 6.

RESULTS

We previously created and characterized an F5 series of βPDGFR mutants consisting of F5, Y1021, Y40/51, Y771, and Y1009 constructs (75). The F5 receptor harbored tyrosine-to-phenylalanine substitutions at the tyrosine phosphorylation sites necessary for binding PLCγ, p85 of PI3K, GAP, and Syp (residues 1021, 740/751, 771, and 1009, respectively) and failed to efficiently associate with all of these receptor-associated enzymes. The other four members of this series efficiently bound only one of the receptor-associated proteins; i.e., Y1021, Y40/51, Y771, and Y1009 receptors associated with PLCγ, PI3K, GAP, and Syp, respectively. We tested the F5 series of receptor mutants for their ability to initiate PDGF-dependent DNA synthesis and found that the F5 receptor was largely unable to send a mitogenic signal and that repairing either the PLCγ- or PI3K-binding sites rescued the majority of the DNA synthesis response. In contrast, restoring GAP- or Syp-binding sites in the F5 receptor failed to rescue the ability of the receptor to initiate DNA synthesis. We have recently found that Src and Grb2 associate with the F5 receptor to near wild-type levels (64); however, this event is not sufficient to elicit a biological response in the HepG2 or TRMP cells (75). These studies demonstrated that the βPDGFR relays a biological signal via its receptor-associated proteins and strongly suggest that PLCγ and PI3K are responsible for initiating mitogenic signal cascades. In contrast, our experiments did not illuminate the role of GAP or Syp in βPDGFR signal relay.

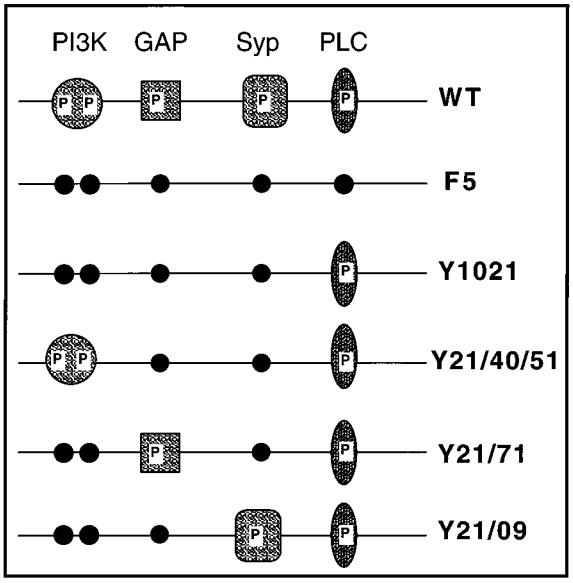


FIG. 1. Schematic of the βPDGFR mutants. The speckled items represent the receptor-associated proteins, and they are labeled at the top of the figure. The P indicates a phosphorylated tyrosine residue, and the solid circle represents a tyrosine-to-phenylalanine substitution. The WT receptor is the wild-type βPDGFR, and the F5 mutant harbors five tyrosine-to-phenylalanine substitutions at positions 740, 751, 771, 1009, and 1021. The names of the other mutants designate which of the phosphorylation sites have been repaired. Y1021 contains tyrosine at position 1021 but phenylalanine at the other four phosphorylation sites. Y21/40/51 has tyrosine at positions 1021, 740, and 751 and phenylalanine at 771 and 1009. In these double-add-back mutants, only the last two numbers of the restored phosphorylation sites are used in the name. The cartoon also indicates which of the receptor-associated proteins are expected to bind to each of the receptor mutants. Additional proteins associate with the activated βPDGFR but are not shown, since their binding sites were not altered in any of the mutants.

One possible function of either Syp or GAP is to regulate the PLCγ pathway that leads to DNA synthesis. To test this theory, we constructed a panel of mutants in which the Y1021 receptor (which associates with PLCγ) was modified to restore the binding site for one additional signaling enzyme, either GAP, Syp, or PI3K. Like the F5 series, the names of the constructs indicate which tyrosines have been repaired; however, only the last two numbers of the repaired tyrosines were used for the name. For instance, the Y21/71 receptor has tyrosine at positions 1021 and 771 and phenylalanine at positions 740, 751, and 1009 and is expected to associate with PLCγ and GAP. Figure 1 illustrates these PLC double add-backs, as well as the proteins predicted to associate with each of the receptor mutants.

The mutant cDNA constructs were generated as described in Materials and Methods, subcloned into the pLXSN retroviral vector (50), and introduced into Ψ2 and then into PA317 virus-producing cells (36). The resulting virus was used to infect HepG2 cells, a human hepatoma cell line that does not express detectable levels of the βPDGFR (75), and mass populations of infected cells were selected on the basis of G418 resistance. Anti-receptor Western blots indicated that all cell lines contained a comparable level of introduced receptor (differences between the cell lines were no more than 2.1-fold), which we have previously estimated as 5×10^5 receptors per cell (75) (see Fig. 3B).

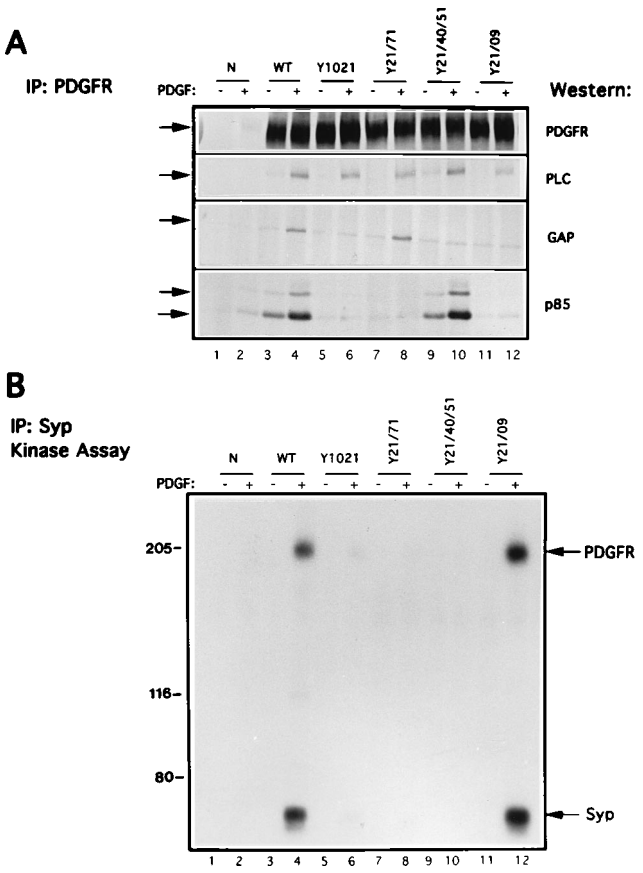


FIG. 2. Characterization of the proteins that associate with the βPDGFR mutants. Confluent, quiescent cultures of HepG2 cells expressing the receptor mutants were left resting (−) or stimulated for 5 min at 37°C with 50 ng of PDGF-BB per ml (+) and then lysed. (A) The lysates were immunoprecipitated with an anti-βPDGFR antibody (30A), immunoprecipitates representing approximately 4.2×10^6 cells were resolved by SDS-PAGE and transferred to Immobilon, and the appropriate portion of the membrane was subjected to Western blot analysis with the antibodies indicated in the right-hand margin; p85 designates the antiserum that recognizes the 85-kDa subunit of PI3K. The arrows in the left-hand margin indicate the major immunoreactive species. (B) The lysates were immunoprecipitated with the anti-Syp antiserum, the resulting immunoprecipitates representing approximately 1.8×10^6 cells were subjected to a standard in vitro kinase assay and resolved by SDS-PAGE, the gel was alkali treated, and the radiolabeled proteins were detected by autoradiography. The PDGFR and Syp bands are indicated by the arrows. The positions of the molecular mass markers (in kilodaltons) are indicated in the left-hand margin. The faint bands in lane 4 correspond to the receptor-associated proteins that associate with the wild-type receptor but not the Y21/09 receptor. The names of the receptor mutants are as in Fig. 1; N refers to cells expressing an empty expression vector.

Characterization of the βPDGFR mutants. To determine the ability of the βPDGFR mutants to associate with PLCγ, GAP, and p85 of PI3K, the βPDGFR was immunoprecipitated from confluent, quiescent cultures of resting or PDGF-stimulated cells, the immunoprecipitates were resolved by SDS-PAGE and transferred to Immobilon, and the appropriate portion of the membrane was subjected to an anti-βPDGFR, anti-PLCγ, anti-GAP, or anti-p85 Western blot (Fig. 2A). The receptor blot showed that there was a comparable level of receptor immunoprecipitated in all of the samples, except the empty vector-expressing cells (lanes 1 and 2), in which there was no receptor detected. The faint band in lane 2 that migrated more slowly than the βPDGFR was not consistently observed. The PLCγ Western blot showed that in response to PDGF stimulation, all of the receptors recruited a similar amount of PLCγ. While all of the samples (even the mock immunoprecipitates in lanes 1 and 2) contained a small amount of GAP, the wild-type and Y21/71 receptors were the only two in which PDGF stimulation promoted efficient binding of GAP. Similarly, p85 was detected at a low level in all samples, but only the receptors with the p85-binding sites (the wild type and Y21/40/51 mutant) showed a robust PDGF-dependent increase in the amount of coimmunoprecipitating p85. As observed previously (75), binding of p85 was somewhat less dependent on PDGF stimulation than was the binding of the other proteins, and this may reflect the fact that the receptor has a small but detectable amount of phosphotyrosine prior to PDGF stimulation (see Fig. 7B). The two immunoreactive species that coprecipitate with the βPDGFR may be distinct forms of p85.

To detect the Syp-PDGFR complex, Syp was immunoprecipitated from resting or PDGF-stimulated cells, the samples were subjected to an in vitro kinase assay, the proteins were resolved by SDS-PAGE, and the radiolabeled proteins were detected by autoradiography (Fig. 2B). Syp and the PDGFR were present in immunoprecipitates from PDGF-stimulated cells expressing either the wild-type or the Y21/09 βPDGFR, while all of the other mutants contained only barely detectable amounts of the receptor-Syp complex. These studies demonstrate that the βPDGFR mutants recruited the receptor-associated proteins predicted in Fig. 1 and that the binding of PLCγ, GAP, p85 of PI3K, and Syp to the βPDGFR depends on the presence of the appropriate tyrosine phosphorylation sites.

PDGF-dependent DNA synthesis. To compare the ability of the βPDGFR mutants to mediate PDGF-dependent DNA synthesis, the receptor-expressing cells were arrested by serum deprivation, stimulated with the indicated concentration of PDGF, and, after 18 to 20 h, pulsed with [^{3}H]thymidine for 2 h and harvested. Consistent with our previous findings, the Y1021 receptor was able to initiate near-wild-type levels of DNA synthesis, whereas the F5 receptor triggered only a marginal response (Fig. 3A) (75). Restoring the Syp-binding site in the Y1021 receptor (the Y21/09 mutant) had little effect, as this receptor initiated DNA synthesis as well as the Y1021 receptor did (Fig. 3A). This mutant (previously called 40/51/71) also exhibited a near-wild-type response when tested in TRMP cells (36). At a saturating dose of PDGF, the Y21/40/51 receptor consistently showed a submaximal DNA synthesis response (Fig. 3A). This was a surprise, given that this receptor associated with PLCγ and PI3K, either of which was able to independently mediate PDGF-dependent DNA synthesis. A possible explanation may involve a competition for substrate, as PI3K can phosphorylate PI-4,5-P_2, making it no longer a substrate for PLCγ (65), whereas PLCγ may deplete PI-4,5-P_2 in certain microenvironments of the cell and thereby reduce the efficiency of PI3K. The response of the Y21/71 mutant (binds PLCγ and GAP) was the poorest in this series, only slightly above the F5 level (Fig. 3A). The differences in the responses do not seem to be related to receptor levels, since there were comparable levels of receptor expressed in all of the cell lines used (Fig. 3B). These studies indicate that PLCγ-dependent initiation of DNA synthesis can be regulated by the other proteins that associate with the βPDGFR.

Activation of PLCγ. What is the mechanism by which certain receptor-associated proteins prevent the Y1021 receptor from triggering DNA synthesis? We reasoned that a possible clue may be provided by considering how the Y1021 receptor is sending a biological signal. The Y1021 receptor activates PLCγ, which then activates a number of enzymes, such as

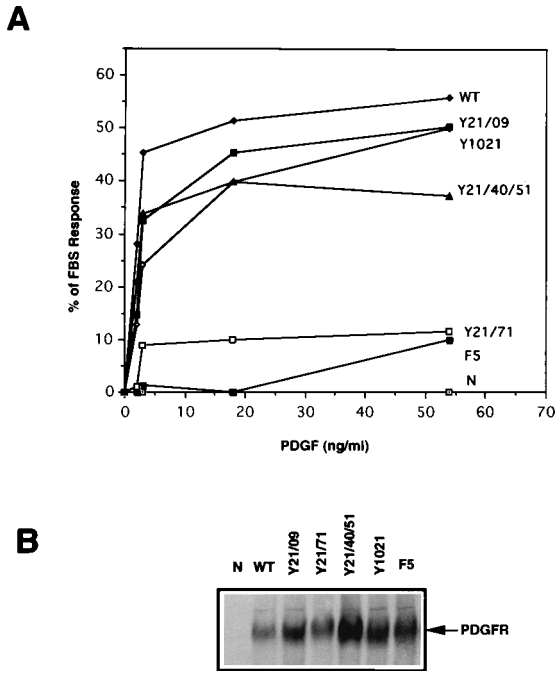


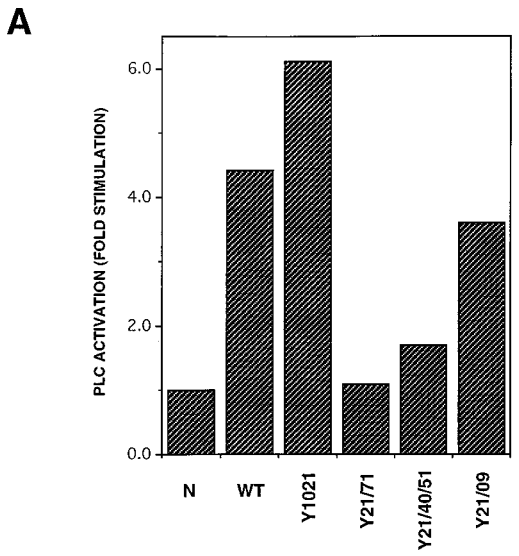
FIG. 3. Capacity of the βPDGFR mutants to initiate DNA synthesis. (A) HepG2 cells expressing the βPDGFR mutants were plated at a subconfluent cell density, arrested by serum deprivation, and stimulated with the indicated concentration of PDGF-BB. After 18 to 20 h, the cells were pulsed with [^{3}H]thymidine for 2 h and harvested, and the radioactivity incorporated was determined. Each point was determined in triplicate, and the replicates were within 19% of each other. While there was some variation in the response of the various mutants at low doses of PDGF (less than 5 ng/ml), the response of the mutants at saturating doses of PDGF was highly reproducible in five independent experiments. The data are expressed as a percentage of the response to 10% FBS; all cell lines typically showed a four- to sixfold response to 10% FBS. (B) Resting HepG2 cells expressing the βPDGFR constructs were lysed, total-cell lysate representing 1.2×10^5 cells was resolved by SDS-PAGE and transferred to Immobilon, and the region of the membrane including 160- to 220-kDa proteins was subjected to a βPDGFR Western blot. The relative receptor levels, as determined by densitometric scanning of the data, were 1.0, 1.5, 1.3, 2.1, 1.7, and 1.7 for the wild-type, Y21/09, Y21/71, Y21/40/51, Y1021, and F5 samples, respectively. The arrow points to the prominent immunoreactive species. The names of the receptor mutants are detailed in the legend to Fig. 1 and 2.

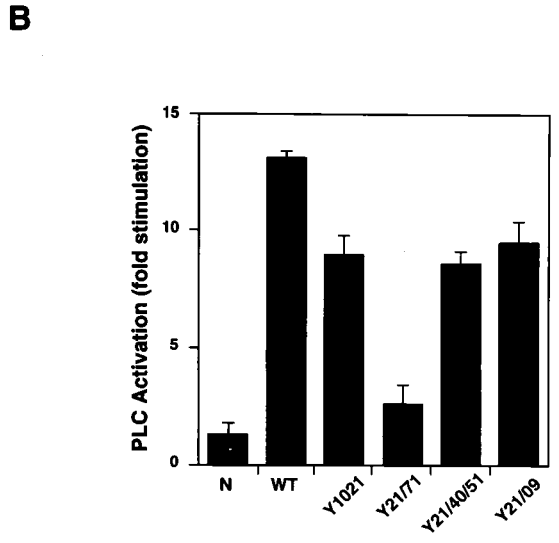
protein kinase C family members, which are implicated in regulating mitogenic signaling cascades (25, 56). Consequently, it is likely that the Y1021 receptor initiates DNA synthesis via a PLCγ pathway. If this is true, the receptor-associated proteins may inhibit Y1021 receptor signaling by preventing PLCγ activation. To test this hypothesis, we compared the ability of the various receptor mutants to mediate PDGF-dependent PLCγ activation in two different assays. In the first approach, we assessed the in vivo activation of PLCγ by measuring PDGF-stimulated accumulation of inositol phosphates in intact cells. Cells were labeled with *myo*-[^{3}H]inositol, exposed to buffer or PDGF, and harvested, and the inositol phosphates were purified on an AG 1-X8 column. As expected, the cells expressing the wild-type and Y1021 receptors, but not an empty expression vector, were able to activate PLCγ (Fig. 4A). The response of the Y1021 receptor was often, but not always (75), slightly greater than that of the wild-type receptor. The Y21/09 receptor initiated close to the wild-type response, the Y21/40/51 receptor was approximately 30% of the maximal response, whereas the Y21/71 receptor consistently failed to drive activation of PLCγ (Fig. 4A).

The second way in which we measured PLCγ activation was to compare the PLCγ activity recovered in antiphosphotyrosine immunoprecipitates. Resting or PDGF-stimulated cells were lysed and immunoprecipitated with an antiphosphotyrosine antibody (1G2 coupled to Sepharose), the immunoprecipitated proteins were released from the immune complex by exposure to 10 mM phenyl phosphate and incubated with [^{3}H]PI-4,5-P_2, and the extent of substrate hydrolysis was determined by counting the amount of ^{3}H released to the aqueous phase. In the wild-type receptor-expressing cells, PDGF-stimulation resulted in a 13-fold increase in the amount of PLCγ activity that was recovered (Fig. 4B). A slightly reduced response was observed in Y1021, Y21/09, and Y21/40/51 receptor-expressing cells, whereas only 20% of the wild-type level (2.6-fold) was recovered from the Y21/71 receptor-expressing cells (Fig. 4B). Little PLCγ activity was immunoprecipitated from the empty-vector-expressing cells, even after PDGF stimulation (Fig. 4B). A PLCγ Western blot of these samples indicated that the amount of activity present in the antiphosphotyrosine immunoprecipitates closely reflected the amount of PLCγ present in the sample (Fig. 4B and C). These studies demonstrate that not all of the receptor mutants that can bind PLCγ are capable of activating it and suggest that some of the receptor-associated proteins function to prevent PLCγ activation. Finally, the finding that PLCγ was poorly immunoprecipitated with the antiphosphotyrosine antibody in the Y21/71 receptor-expressing cells strongly suggests that the mechanism by which the Y21/71 mutant fails to activate PLCγ involves a defect in mediating tyrosine phosphorylation of PLCγ.

Tyrosine phosphorylation of PLCγ. PDGF-dependent activation of PLCγ requires two events: (i) stable association with the βPDGFR and (ii) tyrosine phosphorylation of PLCγ (18, 37, 54, 74). While the Y21/71 receptor is fully able to associate with PLCγ (Fig. 2A), PLCγ is not efficiently immunoprecipitated with an antiphosphotyrosine antibody from cells expressing the Y21/71 receptor mutant (Fig. 4C). Consequently, it appears that the defect in the Y21/71 receptor is at the level of directing PLCγ tyrosine phosphorylation. To directly test this possibility, we compared PDGF-dependent PLCγ tyrosine phosphorylation in the cells expressing the βPDGFR mutants. Resting or PDGF-stimulated cells were lysed, PLCγ was immunoprecipitated, and the immunoprecipitates were analyzed by anti-PLCγ, anti-βPDGFR, and antiphosphotyrosine Western blotting (Fig. 5A). A similar amount of PLCγ was present in each of the PLCγ immunoprecipitates, and a comparable amount of the βPDGFR coimmunoprecipitated with PLCγ from PDGF-stimulated cells. The antiphosphotyrosine Western blot shows that PLCγ was tyrosine phosphorylated in a PDGF-dependent manner in all of the cells and that the Y1021, Y21/40/51, and Y21/09 mutants were able to mediate this event to wild-type levels. In contrast, the Y21/71 receptor initiated PLCγ tyrosine phosphorylation to only 32% of the wild-type level (Fig. 5B). Two-dimensional phosphopeptide maps of PLCγ isolated from PDGF-stimulated cells showed that phosphorylation of all of the PLCγ phosphorylation sites (37) was diminished in the cells expressing the Y21/71 receptor (data not shown). The data in Fig. 2A, 4B, and 5 demonstrate that Y21/71 is fully able to associate with PLCγ but that its ability to direct PDGF-dependent PLCγ tyrosine phosphorylation is severely crippled.

Kinetics of PLCγ tyrosine phosphorylation. It is possible that PLCγ is fully tyrosine phosphorylated in the Y21/71 receptor-expressing cells but at an earlier or later time point relative to the wild-type receptor-expressing cells. To address this issue, we compared the kinetics of PDGF-dependent PLCγ tyrosine phosphorylation in the two different cell types. Confluent, quiescent cells were either maintained at 37°C and





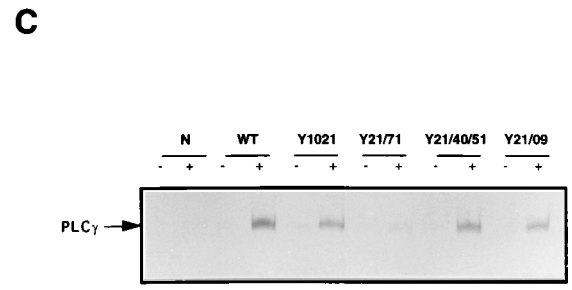
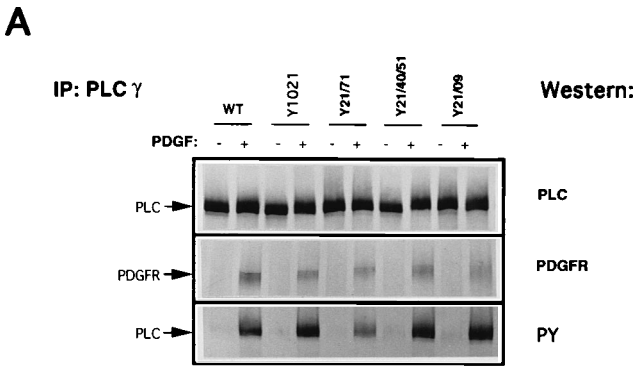


FIG. 4. Activation of PLCγ in HepG2 cells. (A) Confluent, quiescent cultures of HepG2 cells expressing the receptor constructs were labeled with *myo*-[^{3}H]inositol, stimulated for 30 min at 37°C with 200 ng of PDGF-BB per ml in the presence of LiCl, and fixed in acidified methanol, and the total inositol phosphates were purified on an AG 1-X8 column and quantitated by scintillation counting. The data are expressed as fold stimulation, which was calculated by dividing the amount of phospholipids produced in the PDGF-stimulated samples by the amount of phospholipids produced in the resting samples. To calculate the amount of phospholipids produced, the counts per minute eluted from the column were divided into the sum of the counts per minute present in the total cellular phospholipid pool plus the counts per minute eluted from the column. Replicate samples routinely were within 20%, and four independent experiments showed similar results. (B) Confluent, quiescent cultures of HepG2 cells expressing the receptor constructs were left resting or stimulated for 5 min with 100 ng of PDGF-BB per ml and lysed, and the lysates were immunoprecipitated with the 1G2 antiphosphotyrosine antibody coupled to Sepharose beads. Bound proteins were eluted with phenyl phosphate, and 25% of the eluate (representing 5×10^5 cells) was incubated with [2-^{3}H]PI-4,5-P_2-containing vesicles. The water-soluble product ([^{3}H]inositol phosphate) was quantitated by scintillation counting. The results are expressed as the PDGF-dependent fold increase in [^{3}H]inositol phosphate formation. The data presented are the mean values from three independent experiments, and the error bars show the standard error of the mean. (C) Samples eluted from the antiphosphotyrosine immunoprecipitates described in panel B (representing approximately 1.5×10^6 cells) were separated by SDS-PAGE, transferred to Immobilon, and subjected to a PLCγ Western blot. The names of the receptor mutants are detailed in the legends to Fig. 1 and 2.

stimulated with PDGF for 5 min (the standard conditions) or cooled to 4°C and exposed to PDGF for the indicated time. The cells were lysed, PLCγ was immunoprecipitated, and the samples were resolved by SDS-PAGE and subjected to anti-PLCγ and antiphosphotyrosine Western blot analysis. Figure 6A is the Western blot of a representative experiment, and Fig. 6B shows the same data presented as a graph. At the early time points (less than 5 min at 4°C), PLCγ is similarly phosphorylated in cells expressing either receptor, while at the latter time



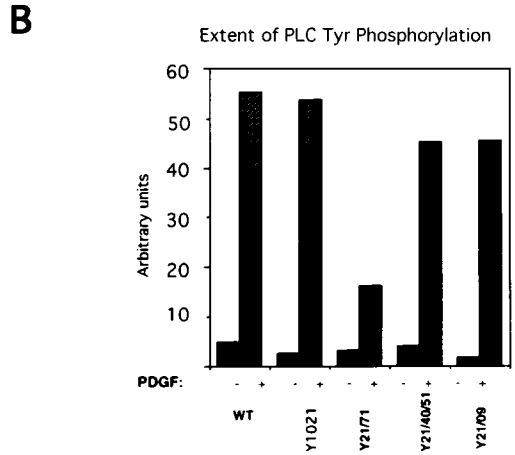


FIG. 5. Tyrosine phosphorylation of PLCγ. Confluent, quiescent cultures of HepG2 cells expressing the βPDGFR mutants were stimulated with 50 ng of PDGF-BB per ml and lysed, and PLCγ was immunoprecipitated. (A) The samples were resolved by SDS-PAGE and transferred to Immobilon, and the appropriate region of the membrane was subjected to a Western blot analysis with anti-βPDGFR and antiphosphotyrosine antibodies, as indicated in the right-hand margin. A portion of the same samples was run on a separate gel and then subjected to a PLCγ Western blot to determine the amount of PLCγ in each of these PLCγ immunoprecipitates. The arrows in the left-hand margin point to the prominent immunoreactive species. (B) The antiphosphotyrosine Western blot in panel A was subjected to densitometric analysis, and the results are presented as a bar graph. The names of the receptor mutants are detailed in the legend to Fig. 1.

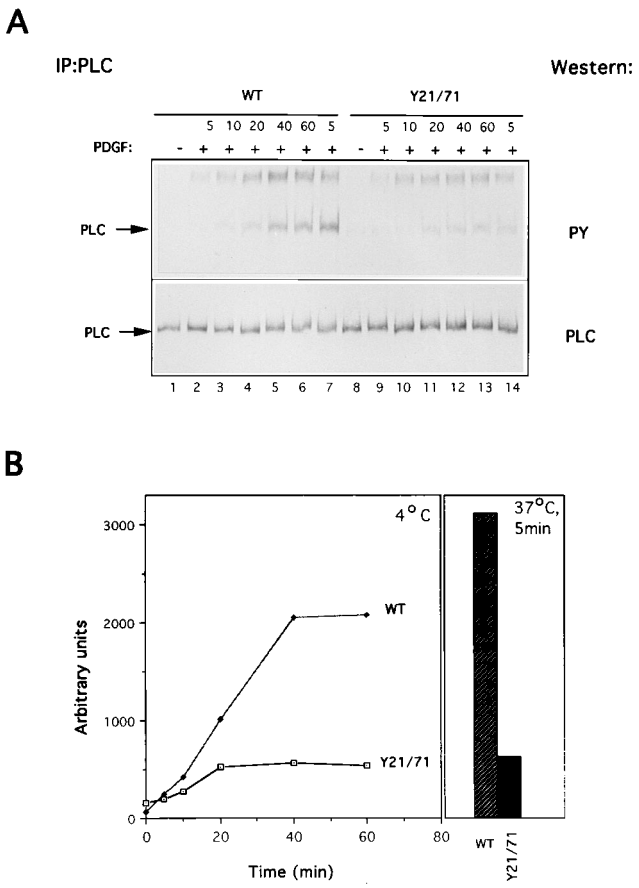


FIG. 6. Kinetics of PLCγ tyrosine phosphorylation. (A) Confluent, quiescent cultures of HepG2 cells expressing the wild-type or Y21/71 receptor were stimulated with 80 ng of PDGF-BB per ml at 37°C for 5 min (lanes 7 and 14) or at 4°C for the time indicated at the top of the figure. The cells were lysed, PLCγ was immunoprecipitated, and the immunoprecipitates were subjected to either an anti-PLCγ (bottom panel) or antiphosphotyrosine (top panel) Western blot. (B) The extent of tyrosine phosphorylation of PLCγ (shown in the top portion of panel A) was quantitated by densitometry and is plotted as a line graph (for the 4°C samples) or a bar graph (for the 37°C samples).

points, PLCγ phosphorylation increases in the cells expressing the wild-type receptor but plateaus at only 26% of the wild-type level in the Y21/71 receptor-expressing cells. The high-molecular-weight species in the antiphosphotyrosine Western blot of Fig. 6A may be the tyrosine-phosphorylated βPDGFR. The lower panel of Fig. 6A is a PLCγ Western blot which shows that there were similar amounts of PLCγ in each of the samples. A time course at 37°C also showed a comparable initial rate by the two receptors (at the 0- to 2-min time points) and a failure to mediate increased PLCγ tyrosine phosphorylation by the Y21/71 receptor-expressing cells at the later time points (greater than 5 min) (data not shown). The kinetics of PLCγ tyrosine phosphorylation in HepG2 cells, driven by the introduced βPDGFR (Fig. 6), were similar to the time course of PLCγ tyrosine phosphorylation by the endogenous PDGFR in NIH 3T3 cells (49). These studies show that the kinetics of PLCγ tyrosine phosphorylation are similar in the wild type and Y21/71 receptor-expressing cells and that the problem with the Y21/71 receptor is that it cannot drive PLCγ tyrosine phosphorylation to the same level as the wild-type receptor can.

Receptor kinase activity of the βPDGFR mutants. A likely explanation for the reduced ability of the Y21/71 receptor to mediate PDGF-dependent PLCγ tyrosine phosphorylation is that this receptor has diminished kinase activity. To test this possibility, we performed a series of experiments to assess the in vitro and in vivo kinase activity of all of the βPDGFR mutants, with a particular emphasis on the Y21/71 receptor.

To compare the in vivo ability of the receptors to autophosphorylate, resting or PDGF-stimulated cells were lysed, total cell lysates were resolved on an SDS-PAGE gel and transferred to Immobilon, and the region of the blot containing proteins of approximately 140 to 220 kDa was subjected to an antiphosphotyrosine Western blot (Fig. 7A). As expected, the cells expressing an empty expression vector failed to respond to PDGF, whereas the wild-type receptor-expressing cells had a prominent PDGF-dependent 180-kDa species (Fig. 7A, lanes 1 to 4). The extent of PDGF-dependent tyrosine phosphorylation of all the mutant receptors was comparable to the wild-type level (lanes 4 to 12). Previous studies have indicated that the WT and F5 receptors are tyrosine phosphorylated to a similar extent in PDGF-stimulated cells (27). Thus, the various mutations did not affect the PDGF-activated, in vivo autophosphorylation response of the βPDGFR.

Since the Y1021, Y21/40/51, and Y21/09 mutants were able to (i) mediate wild-type levels of PLCγ tyrosine phosphorylation (Fig. 5), (ii) autophosphorylate in vivo (Fig. 7A) and in vitro (data not shown), and (iii) phosphorylate an exogenous substrate in vitro (data not shown), we concluded that the kinase activity of these receptors is not damaged. We then focused our efforts on determining the integrity of the Y21/71 receptor kinase activity.

To compare the phosphorylation of a panel of in vivo substrates, resting or PDGF-stimulated cells expressing the wild-type or Y21/71 receptors were lysed and immunoprecipitated with an anti-βPDGFR, anti-GAP, or anti-pp120 antibody, and the immunoprecipitated proteins were subjected to antiphosphotyrosine Western blot analysis. In the anti-receptor immunoprecipitates, the receptor and GAP were readily detected and both proteins were phosphorylated to a similar extent in the Y21/71 and wild type receptor-expressing cells (Fig. 7B, lanes 1 to 4). In anti-GAP immunoprecipitates, a comparable amount of tyrosine-phosphorylated GAP was detected in the wild-type and mutant samples (lanes 5 to 8). Consistent with Fig. 2A and 7A, a similar amount of tyrosine-phosphorylated PDGFR coimmunoprecipitated with GAP from cells expressing the wild-type or Y21/71 receptor. The species running just below the PDGFR (denoted with a black oval to the right of the figure) is most probably the 190-kDa GAP-associated protein (14, 66). We did not detect tyrosine phosphorylation of p62 (data not shown), a GAP-associated protein that is tyrosine phosphorylated in response to epidermal growth factor stimulation or transformation with activated tyrosine kinases (7, 14, 80). The pp120 protein was originally described as a Src substrate (59) and has subsequently been shown to be phosphorylated in response to growth factor stimulation of cells (12a, 28). It was tyrosine phosphorylated in PDGF-stimulated HepG2 cells that expressed the wild-type, F5, or Y21/71 receptors (Fig. 7B lanes 12 to 20). In contrast, there was no detectable phosphorylation of pp120 in cells expressing an empty expression vector (lanes 9 to 11). This series of experiments shows that the Y21/71 receptor is indistinguishable from the wild-type receptor in its ability to mediate tyrosine phosphorylation of the PDGFR, GAP, and pp120 in vivo.

We also compared the ability of the Y21/71, F5, and wild-type receptors to autophosphorylate and to phosphorylate exogenous substrates in vitro. One of the exogenous substrates tested was a GST-PLCγ fusion protein that encompassed the PLCγ phosphorylation sites at positions Y771 and Y783. This

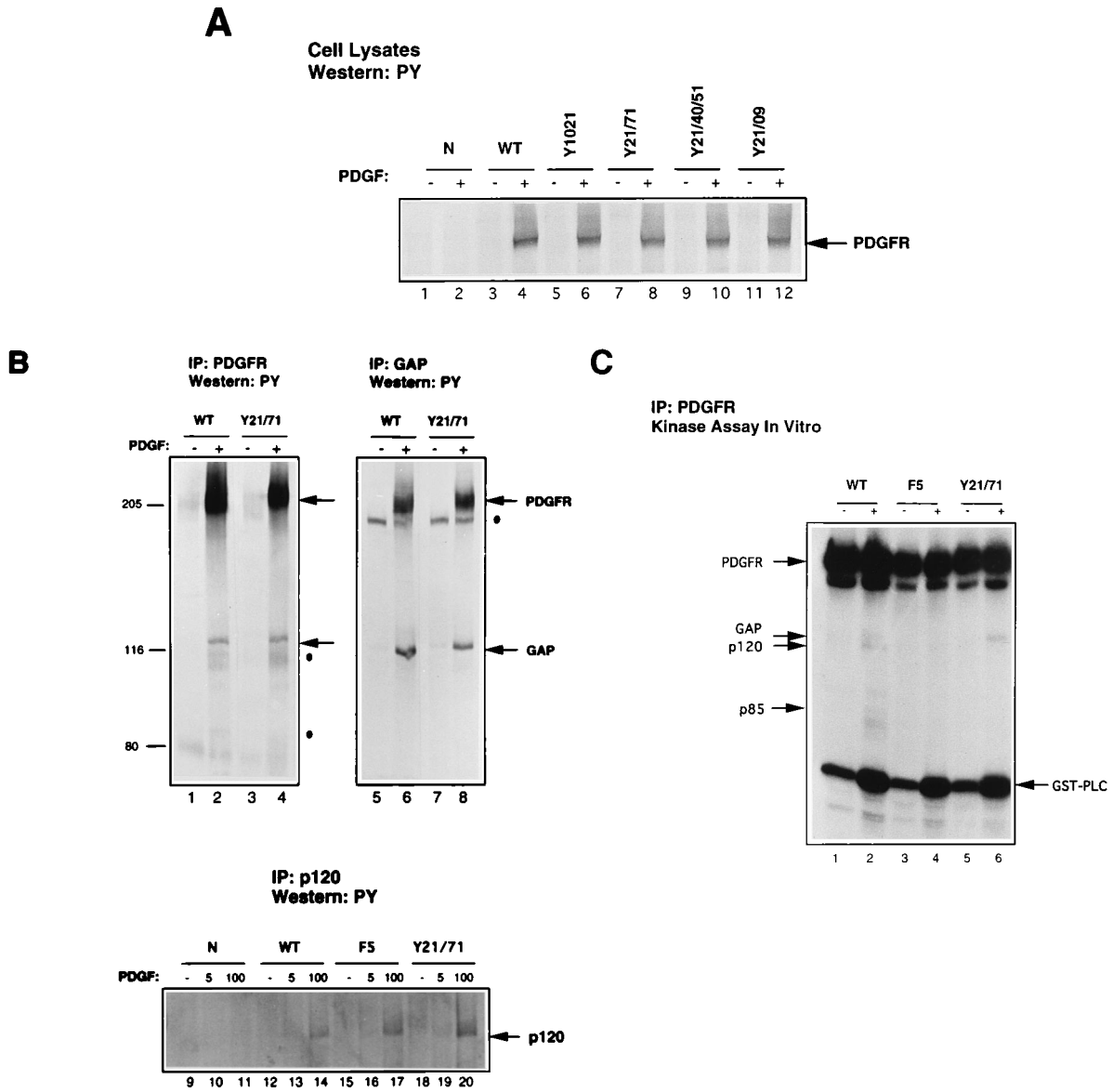


FIG. 7. Kinase activity of the βPDGFR mutants. Confluent, quiescent cultures of HepG2 cells expressing the βPDGFR mutants were left resting (−) or stimulated (+) for 5 min with 50 ng (unless otherwise indicated) of PDGF-BB per ml and then lysed. (A) Total-cell lysates representing 6×10^5 cells were resolved by SDS-PAGE and transferred to Immobilon, and the region of the membrane containing the 140- to 220-kDa proteins was subjected to an antiphosphotyrosine Western blot. (B) The lysates were immunoprecipitated with either anti-βPDGFR, anti-GAP, or anti-p120 antibodies and subjected to an antiphosphotyrosine Western blot analysis. The black ovals to the right of the left panel designate the positions of the 120-kDa and p85 receptor-associated proteins that associate with the wild-type but not the Y21/71 receptor. The black oval to the right of the right panel indicates the position of the 190-kDa GAP-associated protein. The arrows mark the position of GAP and the PDGFR. In the lower panel, 5 and 100 indicate the amount of PDGF (in nanograms per milliliter) that was used to stimulate the cells. (C) The lysates were immunoprecipitated with PR7212 (a monoclonal anti-βPDGFR antibody recognizing an extracellular epitope). Immunoprecipitates representing approximately 7×10^5 cells were subjected to a standard in vitro kinase assay modified to include 0.5 μg of the GST-PLC fusion protein. The samples were resolved on an SDS-PAGE gel, and the radiolabeled proteins were detected by autoradiography. The positions of the 61-kDa exogenous substrate (GST-PLC) and several of the receptor-associated proteins is indicated. The names of the receptor mutants are detailed in the legend to Fig. 1 and 2.

substrate was used not only to examine the overall kinase activity of the receptors but also to identify possible changes in the substrate specificity of the Y21/71 receptor mutant. PDGFRs were immunoprecipitated from resting or PDGF-stimulated cell lysates and subjected to an in vitro kinase assay in the presence of the exogenous substrate. Following the kinase assay, the proteins were resolved by SDS-PAGE and the radiolabeled proteins were detected by autoradiography. Comparison of the kinase activity from resting and PDGF-stimulated samples revealed that the samples prepared from PDGF-stimulated cells had an enhanced ability to phosphorylate the PLCγ fusion protein (Fig. 7C). In addition, there was no apparent difference in the extent of substrate phosphorylation between the wild-type, F5, and Y21/71 receptors (Fig. 7C). Finally, we also tested the ability of the Y21/71 receptor, as well as all of the other mutants in this series, to phosphorylate a second substrate, a GST fusion protein including the C terminus of the αPDGFR (75), and found that all of the receptors

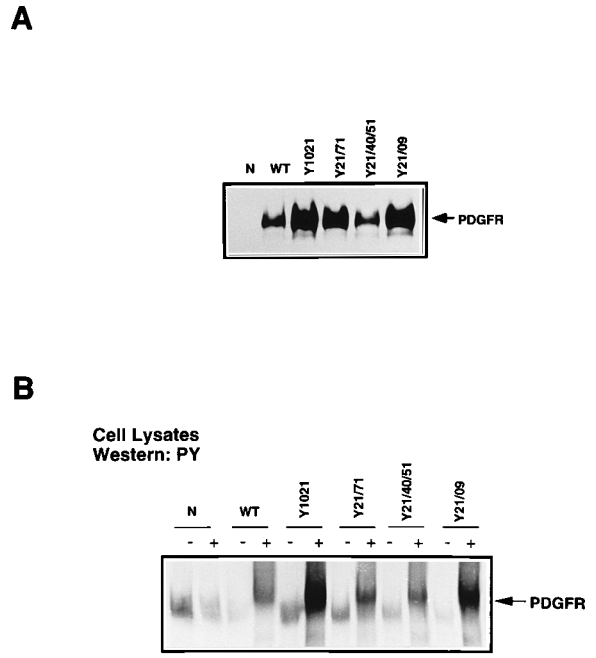


FIG. 8. Characterization of the A431 cells expressing the βPDGFR mutants. (A) Resting cultures of A431 cells expressing the various βPDGFR mutants were lysed, and lysate representing approximately 1.2×10^5 cells was subjected to an anti-βPDGFR Western blot. The arrow points to the mature receptor, whereas the faint, lower-molecular-weight species is probably the immature form of the receptor. (B) Total-cell lysates from resting (−) and PDGF-stimulated (+) (50 ng of PDGF-BB per ml for 5 min) cells representing 6×10^5 cells were analyzed by antiphosphotyrosine Western blotting. The names of the receptor mutants are detailed in the legend to Fig. 1 and 2.

were equally able to phosphorylate this protein (data not shown).

These studies demonstrate that the mutant βPDGFRs do not differ from the wild-type receptor in their overall in vitro or in vivo kinase activity. Note that this observation is consistent with our previous findings that the F5 and Y1021 receptors, which have two or three more tyrosine-to-phenylalanine substitutions than do the Y21/71 receptors, have normal receptor kinase activity (75). Finally, the reduced ability of the Y21/71 receptor to direct phosphorylation of PLCγ in vivo does not seem to be due to a reduced substrate specificity for PLCγ, as this receptor is fully capable of phosphorylating the PLCγ fusion protein in vitro.

A431 cells. To assess the possibility that the behavior of these βPDGFR mutants in HepG2 cells was unique to this cell type, we expressed this series of mutants in the human epidermoid carcinoma cell line A431. These cells have been previously reported to lack PDGFRs (23), and, consistent with these reports, we were unable to detect either αPDGFR or βPDGFR by Western blot analysis or immunoprecipitation/in vitro kinase assays (Fig. 8A) (data not shown). The A431 cell line was infected with the appropriate βPDGFR virus or a virus harboring the empty expression vector, and mass populations of cells were selected on the basis of resistance to G418. Anti-βPDGFR Western blot analysis of total cell lysates prepared from the G418-selected cells showed that the introduced receptor was expressed at comparable levels, although somewhat higher in the Y1021 and Y21/09 cell lines (Fig. 8A).

To further characterize the receptor-expressing cells, we determined if the introduced receptors would autophosphorylate in response to PDGF stimulation. Resting or PDGF-stimulated cells were lysed, and the total cell lysate was subjected to an antiphosphotyrosine Western blot. The cells expressing the wild-type receptor or any of the mutants demonstrated a very prominent, PDGF-dependent tyrosine-phosphorylated species of 180 kDa (Fig. 8B). The greater level of phosphorylated receptor in the Y1021 and Y21/09 cell lines reflects the higher level of receptor expression in these cells (Fig. 8A). In contrast, the empty-vector-expressing cells did not respond to PDGF. All of the samples contained an approximately 170-kDa protein that may be the epidermal growth factor receptor, which is highly overexpressed in this cell line. In addition, the ability of this panel of PDGFR mutants to recruit the various receptor-associated proteins was identical when expressed in either A431 or HepG2 cells, with the exception of Syp binding, which displayed slightly less of a dependence on tyrosine 1009 in the A431 cells (data not shown).

We next compared the capacity of the expressed receptors to activate PLCγ in vivo. Cells were labeled with *myo*-[^{3}H]inositol and were left resting or stimulated with PDGF, and the inositol phosphates were extracted and purified, as was done with the HepG2 cells in Fig. 4A. The cells expressing the wild-type βPDGFR, but not an empty expression vector, showed a robust, PDGF-dependent activation of PLCγ (Fig. 9A). As in the HepG2 cells, the response of the cells expressing the Y1021 and Y21/09 receptors was comparable to the wild-type level. The Y21/40/51 receptor was also able to initiate a near-wild-type PLCγ activation (Fig. 9A), whereas in the HepG2 cells this receptor consistently showed a suboptimal response (Fig. 4A). Hence, the reduced ability of the Y21/40/51 receptor to initiate PLCγ activation is cell type dependent. As in the HepG2 cells, the Y21/71 receptor displayed a severely reduced ability to activate PLCγ in the A431 cells (26% of the wild-type level [Fig. 9A]).

To determine whether the inability of the Y21/71 receptor to activate PLCγ correlated with the failure to mediate PLCγ tyrosine phosphorylation, as it did in HepG2 cells, we compared the extent of PLCγ tyrosine phosphorylation in the A431 cells expressing the various PDGFR mutants. Resting and PDGF-stimulated cells were lysed, PLCγ was immunoprecipitated, and the immunoprecipitates were subjected to anti-PLCγ or antiphosphotyrosine Western blot analysis. PLCγ was tyrosine phosphorylated to a comparable extent following PDGF stimulation of A431 cells expressing the wild-type, Y1021, Y21/40/51, and Y21/09 receptors (Fig. 9B). In contrast, tyrosine phosphorylation of PLCγ was barely detectable in the A431 cells expressing the Y21/71 receptor (Fig. 9B). This observation is similar to the situation in HepG2 cells, in which only the Y21/71 receptor was unable to mediate wild-type levels of PLCγ tyrosine phosphorylation (Fig. 5). The lower panel of Fig. 9B is a PLCγ Western blot showing that there were comparable amounts of PLCγ in all of the samples. These experiments show that in both HepG2 and A431 cells, the Y21/71 βPDGFR mutant fails to efficiently tyrosine phosphorylate or activate PLCγ.

DISCUSSION

We have constructed and characterized a panel of βPDGFR mutants which were able to associate with PLCγ alone, PLCγ and PI3K, PLCγ and GAP, or PLCγ and Syp. Despite their uniform ability to recruit PLCγ, the receptors differed dramatically in their ability to activate PLCγ and to initiate DNA synthesis. The underlying mechanism involved suppression of tyrosine phosphorylation of PLCγ, without a detectable change in the kinase activity or substrate specificity of the receptor. The mutant receptor least able to activate the PLCγ

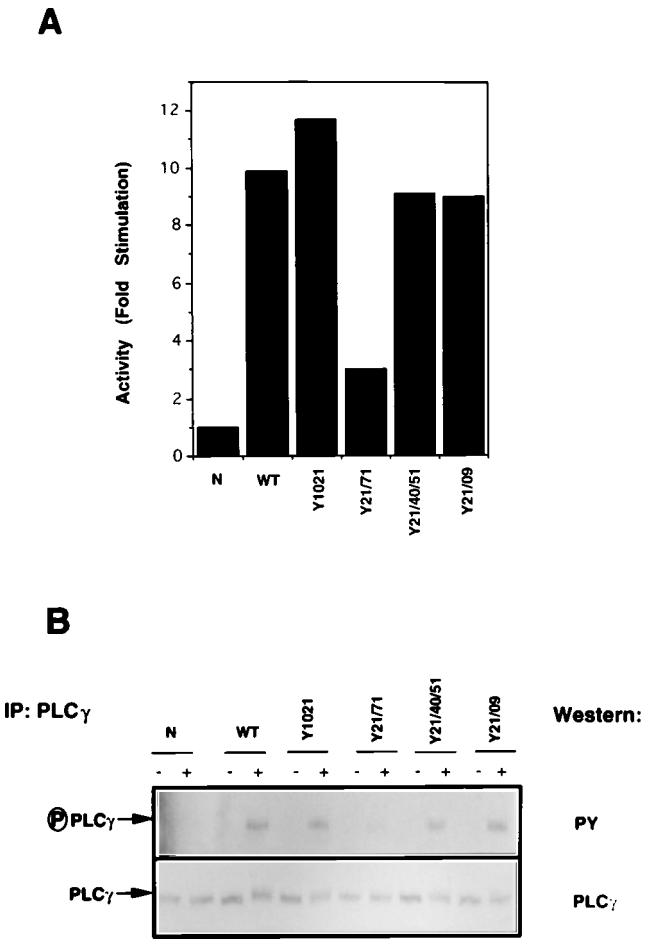


FIG. 9. Activation and tyrosine phosphorylation of PLCγ in A431 cells expressing the mutant βPDGFRs. (A) Confluent, quiescent cells were labeled with *myo*-[^{3}H]inositol and stimulated with PDGF-BB (70 ng/ml), and production of total inositol phosphates was determined as described in the legend to Fig. 4. The data presented are fold stimulation, which was calculated by dividing the response of PDGF-stimulated cells by the response of unstimulated cells. The variation between individual datum points was routinely no more than 9%, and four independent experiments gave similar results. (B) Confluent cultures of cells were left unstimulated or exposed to 50 ng of PDGF-BB per ml for 5 min at 37°C, the cells were lysed, PLCγ was immunoprecipitated, and the immunoprecipitates were analyzed by anti-PLCγ (lower panel) or antiphosphotyrosine (upper panel) Western blot analysis. The names of the receptor mutants are detailed in the legend to Fig. 1 and 2.

pathway was Y21/71, suggesting that GAP functions to silence PLCγ. These studies reveal the previously unappreciated ability of GAP to regulate the tyrosine phosphorylation state of PLCγ and thereby to regulate activation of PLCγ and signaling by the βPDGFR. Furthermore, these studies indicate that the composition of the proteins that associate with the βPDGFR determines the capacity of the receptor to signal.

It is possible that GAP is not the protein responsible for the observed effects on PDGFR signaling but that some other protein can bind to the receptor GAP-binding site. A number of groups have looked for receptor-associated proteins whose binding depends on tyrosine 771 phosphorylation. Mutating tyrosine 771 to phenylalanine results in the selective loss of GAP binding, whereas PLCγ, PI3K, Syp, Grb2, Src, and several as yet unidentified proteins associate with the receptor normally (references 15, 29, 36, and 75 and unpublished data). If there are other proteins that compete with GAP for binding to the receptor at the Y771 site, depleting cells of GAP should increase the recovery of such proteins. This idea was tested by comparing the receptor-binding proteins retrieved from control or GAP-depleted lysates in an in vitro binding assay (34). While removing GAP did abolish GAP binding, no new proteins were detected in an in vitro kinase assay (unpublished observations). Thus, while it is virtually impossible to unequivocally determine that GAP is the only protein which associates with the receptor in response to phosphorylation of tyrosine 771, numerous attempts to detect such proteins have not been successful.

A related question is the importance of PLCγ activation for PDGF-dependent initiation of DNA synthesis in Y1021 receptor-expressing cells. Since the Y1021 receptor activates Ras (75), it is possible that Ras activation is the key event leading to entry into the cell cycle. This seems unlikely because we have found the Y1009 receptor is able to activate Ras but cannot initiate DNA synthesis (75). Thus, while the relatively small change in the level of active Ras may be an important event in PDGF-dependent initiation of DNA synthesis, it is not sufficient in the HepG2 cell system. In contrast, there is a good correlation between activation of PLCγ and initiation of DNA synthesis in our model system. Ongoing studies are focused on unraveling the mitogenic pathway initiated by the Y1021 receptor and, in particular, on defining the relative role of PLCγ.

We have focused on the binding of PI3K, PLCγ, Syp, and GAP to the receptor mutants described in these experiments; however, it is likely that other proteins associate with these receptors as well. For instance, we have recently found that Src and Grb2 associate with the F5 receptor to near wild-type levels (unpublished observations). The F5 receptor is unable to initiate DNA synthesis in two different cell lines (HepG2 and TRMP) (Fig. 3) (75); indicating that binding of Grb2 and Src (and possibly of other proteins as well) is insufficient to relay a mitogenic response in these cell types. However, it is possible that these other receptor-associated proteins in some way contribute to the responses shown in these studies. For instance, binding of Src may be important for activation of the receptor kinase activity, as mutant βPDGFRs that do not associate with detectable levels of Src display severely impaired kinase activity (reference 51 and unpublished data) and injection of kinase inactive Src or Fyn DNA constructs blocks signaling by the βPDGFR (73).

Inhibition of the PLCγ pathway. Studies with other cell surface receptors have indicated that PLCγ can be negatively regulated by serine phosphorylation. In the Jurkat T-cell line, activation of the T-cell receptor results in tyrosine phosphorylation and activation of PLCγ, and these events can be prevented by the stimulation of cells with activators of protein kinase C or protein kinase A (55). The mechanism appears to involve phosphorylation of PLCγ at serine 1248. This does not seem to be a universal mode of PLCγ regulation, since protein kinase C agonists do not affect PLCγ activation in peripheral T cells or in PDGF-stimulated fibroblasts (22, 77). In addition, PDGF stimulation of fibroblasts activates protein kinase C and causes phosphorylation of serine 1248, yet PLCγ is tyrosine phosphorylated and activated (37). Phosphopeptide maps indicated that PDGF-stimulated serine phosphorylation of PLCγ was comparable in cells expressing the various βPDGFR mutants (unpublished observations). Thus, the variable capacity of the receptor mutants to activate PLCγ was not related to gross differences in PDGF-stimulated serine phosphorylation of PLCγ.

While the Y21/71 receptor was the least able to activate PLCγ, the Y21/40/51 receptor also failed to initiate a wild-type

7response in HepG2 cells (Fig. 3 and 4). Unlike the Y21/71 receptor, the problem with the Y21/40/51 receptor was not at the level of PLCγ tyrosine phosphorylation (Fig. 4B, 5, and 7), indicating that PLCγ activation proceeded normally. The problem may lie at the level of substrate availability. The Y21/40/51 receptor recruits and activates PLCγ and PI3K, both of which use PI-4,5-P_2 as a substrate. Consequently, it is possible that PI3K reduces the available pool of substrate for PLCγ, which attenuates the PLCγ mediated signal. An alternate possibility is that the Y21/40/51 receptor is at the cell surface for a shorter period than the other receptors in this series, since the Y21/40/51 receptor binds PI3K, which appears to direct trafficking of the receptor (27). Furthermore, the availability of PI-4,5-P_2 and/or the activity of PLCγ may not be identical at the plasma membrane and in an endosome. Note that the diminished capability of the Y21/40/51 receptor to activate PLCγ was cell type specific, as this receptor mediated a wild-type response when expressed in A431 cells (Fig. 9A). Our studies with these receptor mutants indicate that there are multiple mechanisms by which PLCγ activation is regulated and that the PLCγ response depends on the cell type for some of the mutants studied.

A likely explanation for the inability of the Y21/71 receptor to drive efficient tyrosine phosphorylation of PLCγ is that the mutations in this receptor have reduced its kinase activity and/or changed its substrate specificity. Extensive studies examining PDGF-dependent phosphorylation of proteins in vivo and in vitro show that the kinase activity of the Y21/71 and wild-type receptors are indistinguishable (Fig. 7). In addition, the F5 receptor, which has two additional tyrosine-to-phenylalanine changes, has normal kinase activity (Fig. 7) (75). Consequently, some other feature of the Y21/71 receptor is responsible for its reduced ability to activate PLCγ. Comparison of the Y21/71 receptor with the other mutant receptors in this series indicates that binding of GAP tightly correlates with the reduction in ability to mediate PLCγ tyrosine phosphorylation.

The implication that GAP regulates the level of tyrosine phosphorylated PLCγ is surprising since GAP and its associated proteins, p190 and p62, do not share any obvious homology with phosphatases (66, 71, 76, 80), and none of these proteins have been reported to have phosphatase activity. Recent studies show the p62 GAP-associated protein stably associates with PLCγ in EGF-stimulated or v-*src*-transformed cells (43), suggesting that there is a connection between GAP and PLCγ. One of the known functions of GAP is to regulate the level of RasGTP; however, this event is not currently thought to be involved with PLCγ tyrosine phosphorylation. Consequently, it is likely that GAP blunts PLCγ tyrosine phosphorylation by acting via an intermediary protein. One of the known functions of SH2 and SH3 domain-containing proteins is to act as adapters; for instance, recent studies show that tyrosine phosphorylation of Syp enables it to stably associate with Grb2 and that in certain cell types Grb2 binds to the βPDGFR via Syp (2, 41). By analogy, it is possible that GAP associates with a phosphatase, and binding of GAP to the receptor enhances access of this phosphatase to tyrosine-phosphorylated PLCγ. The allure of this hypothesis is diminished by the observation that the Y21/09 receptor, which binds PLCγ and Syp, shows wild-type levels of PLCγ tyrosine phosphorylation.

An alternate explanation of why the Y21/71 receptor inefficiently mediates PLCγ tyrosine phosphorylation is that PLCγ is tyrosine phosphorylated by a kinase that can be efficiently sequestered and/or repressed by GAP. While PLCγ can be phosphorylated by the PDGFR in vitro (49), Src family members also mediate this in vitro event (42), and the kinase responsible for phosphorylating PLCγ in PDGF-stimulated cells has not been unequivocally identified. Furthermore, PDGF stimulation activates multiple kinases that include Src family members (19, 40, 58). In certain systems, such as the T-cell receptor, in which the receptor itself is not a tyrosine kinase, Src family members are responsible for tyrosine phosphorylation of PLCγ (79). These observations raise the possibility that Src or some other PDGF-activated tyrosine kinase contributes to phosphorylation of PLCγ in a PDGF-stimulated cell. This possibility is also supported by the observation that PLCγ complexes with Src family members (52, 78). Importantly, GAP has also been shown to associate with tyrosine kinases, which include members of the Src family (5, 9, 24, 57). Thus, GAP may be blunting PLCγ tyrosine phosphorylation by sequestering Src or some other kinase that phosphorylates PLCγ in a PDGF-stimulated cell.

There are other examples of GAP negatively regulating signaling by receptor tyrosine kinases. Development of the R7 cell in the *Drosophila* eye requires activation of the Sevenless (Sev) receptor tyrosine kinase (20, 38, 69, 70). A locus was identified that eliminated the need for functional Sev, and sequence analysis revealed a gene with high homology to the catalytic domain of GAP-like proteins (17). Since the Sev signal relay pathways involve activation of Ras (16), it appears that GAP functions to suppress Sev signaling by inactivating Ras. In contrast, the mechanism by which GAP negatively regulates receptor signaling in mammalian cells does not appear to involve suppression of Ras activation. The role of GAP in PDGF-dependent Ras activation has been tested in a variety of cell lines by several groups, and in none of these studies was binding of GAP to the receptor found to be important for activation of Ras (63, 75). In addition, our preliminary studies show that the wild-type and Y21/71 receptors are equally capable of activating Ras (unpublished observations). These results are consistent with the findings that receptor tyrosine kinases in mammalian cells activate Ras by regulating nucleotide exchange factors instead of GAP activity (6, 53, 83). Thus, while a common function of GAPs is to attenuate signaling by growth factor receptors, there appear to be at least two distinct ways in which GAPs accomplish this task.

Activation of PLCγ by the wild-type receptor. The wild-type receptor associates with GAP and PLCγ, as does the Y21/71 receptor; however, the wild-type receptor is fully able to activate the PLCγ pathway. There are many explanations for this observation. For instance, there may be additional proteins that bind to the wild-type receptor that are able to alleviate the inhibition of the PLCγ pathway. Alternatively, the difference in the biological output of the wild-type and Y21/71 receptors relates to the exact mixture of GAP and PLCγ within the receptor dimer. For instance, the Y21/71 receptor, which has a fully active kinase but is missing three tyrosine phosphorylation sites, may phosphorylate both the GAP- and PLCγ-binding sites on a single receptor more often than does the wild-type receptor, which has the same kinase activity but more sites to phosphorylate. This idea is supported by the observation that the extent of receptor autophosphorylation is similar in the wild-type and Y21/71 receptors (Fig. 7A). Consequently, in comparison with the wild-type receptors, the Y21/71 receptor population would have more receptor molecules associated with both PLCγ and GAP. When GAP cannot associate with the wild-type receptor (as in the F771 receptor mutant), there is not a dramatic increase in PDGF-stimulated PLCγ activation (unpublished observations), which is the expected result if only a minority of the wild-type receptors associate with both PLCγ and GAP. We have recently found that PLCγ is activated normally in cells coexpressing the Y1021 and Y771

(binds only GAP) receptors (unpublished observations), which further supports the hypothesis that GAP must bind to the same receptor that associates with PLCγ in order to block signaling. Strong support for the idea of receptors bearing distinct sets of receptor-associated proteins is provided by the observation that the stoichiometry of phosphorylation of the various sites on the receptor is not uniform (29, 33). Consequently, it is highly likely that a PDGF-activated cell contains receptors with distinct tyrosine residues phosphorylated and hence different sets of receptor-associated proteins. If one of the cellular functions of GAP is to sequester a kinase responsible for phosphorylating PLCγ, then binding to the same receptor that associates with PLCγ may increase the ability of GAP to carry out this function.

Under what circumstances would the wild-type receptor need the ability to negatively regulate the intensity of its mitogenic signal? Studies comparing signal relay pathways used by cells engaged in a proliferative versus differentiation response have revealed surprisingly few differences (reviewed in reference 8). Assuming that all of the relevant players in these signaling pathways have been identified, then the distinct response must arise from subtle differences in the qualitative or quantitative use of these signaling molecules. In some instances, the same receptor is able to initiate both of these biological responses, and the difference lies in the level of expression of the receptor (12, 72). The studies described herein add a variation to this concept, i.e., that the interaction among the various receptor-associated proteins can attenuate signals and that this may be responsible for the switch between differentiation and proliferation.

The data presented clearly show that the proteins that are recruited to the receptor have multiple functions which include not only the initiation of a signaling pathway that can start the cell cycle but also the attenuation of such signals. In addition, these studies provide a second example of GAP acting to negatively regulate signaling by receptor tyrosine kinases, and they identify a novel mechanism by which inhibition of signaling is accomplished. Finally, these experiments reveal that signaling by the βPDGFR depends not only on the ability to recruit effectors but also on the blend of these receptor-associated proteins.

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