The Platelet-derived Growth Factor β Receptor Triggers Multiple Cytoplasmic Signaling Cascades That Arrive at the Nucleus as Distinguishable Inputs*

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Stimulation of the platelet-derived growth factor β receptor (βPDGFR) activates enzymes such as phosphatidylinositol 3-kinase (PI3K) and phospholipase Cγ1 (PLCγ), which ultimately initiate nuclear responses such as enhanced expression of immediate early genes. In an attempt to compare the signaling cascades initiated by PI3K and PLCγ, we examined the activation of a panel of immediate early genes by βPDGFR mutants, which preferentially engage PI3K or PLCγ. When expressed in A431 cells, the wild type receptor and to a lesser extent the mutant receptor that associates with PLCγ (Y1021) was able to up-regulate c-fos, junB, and KC mRNA expression. In contrast, the receptor mutant that engages PI3K (Y740/51) poorly stimulated c-fos mRNA expression and did not significantly stimulate expression of either JunB or KC. Receptor mutants that did not associate with either PI3K or PLCγ were dramatically compromised or unable to increase expression of any of these immediate early genes. The differential ability of the Y1021 and Y740/51 receptors to activate c-fos correlated well with an apparent difference in their ability to engage distinct protein kinase C family members. However, there did appear to be a degree of redundancy in the cytoplasmic signaling pathways initiated by PI3K and PLCγ, since both the Y1021 and Y740/51 receptors were able to activate an AP-1-responsive element. We conclude that recruitment of signal relay enzymes to the βPDGFR is necessary for PDGF-dependent activation of at least some immediate early genes. In addition, whereas the βPDGFR activates multiple signaling enzymes capable of activating the same nuclear response (activation of c-fos), these signaling cascades do not appear to converge in the cytoplasm but arrive at the nucleus as distinguishable inputs.

The receptor for platelet-derived growth factor (PDGF)1 is a transmembrane protein with a tyrosine kinase domain within the intracellular portion of the receptor (1). The receptor is a ligand-inducible dimer, and there are two different receptor subunits, α and β, that can participate in the formation of a functional receptor (2). Many tissue culture cells express both PDGF receptor subunits, and the type of PDGF (AA, BB, or AB) determines the subunit composition of the PDGF receptor dimer (2, 3). The studies presented here are restricted to the PDGF β receptor (βPDGFR), which dimerizes in response to binding PDGF-BB.

Ligand binding to the receptor initiates many intracellular events including receptor kinase activation and phosphorylation of the receptor at up to nine different tyrosine residues (2, 4). The functional consequences of receptor tyrosine phosphorylation include activation of the receptor kinase activity and creation of binding sites for numerous SH2 domain-containing proteins. The list of SH2 domain-containing proteins that associate with the receptor includes signaling enzymes such as phospholipase Cγ1 (PLCγ), phosphatidylinositol 3-kinase (PI3K), the GTPase-activating protein of Ras (RasGAP), Src family members, and the phosphotyrosine phosphatase SHP-2 (previously called Syp, SH-PTP2, PTP1D, and others) as well as adaptor proteins such as Shc, Nck, Grb7, and Grb2 (2, 4, 5).

As the number of βPDGFR-associated proteins has grown, questions concerning their relative contribution to βPDGFR signal relay have arisen. Characterization of βPDGFR mutants that are unable to associate with at least some of these SH2 domain-containing proteins revealed that either PI3K or PLCγ is required for PDGF-stimulated DNA synthesis in epithelial cells (6). The use of a microinjection approach to evaluate the importance of proteins that associate with the βPDGFR for signal relay has indicated that most of the proteins that associate with the receptor are required for cell cycle progression (7–10).

These studies clearly indicate that the SH2 domain-containing signal relay enzymes are important for PDGF-mediated signaling and demonstrate that the receptor is able to initiate multiple signaling cascades leading to the same nuclear response (induction of immediate early genes or progression through the cell cycle). Such observations raise the question of the degree of redundancy in receptor signaling: do all of the signaling pathways that trigger a given nuclear response converge at some point before arriving at the nucleus, or alternatively, does each signaling pathway send a unique signal to the

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1 The abbreviations used are: PDGF, platelet-derived growth factor; βPDGFR, PDGF β receptor; PLC, phospholipase C; PI3K, phosphatidylinositol 3-kinase; RasGAP, GTPase-activating protein of Ras; AP-1, activator protein; CAT, chloramphenicol acetyltransferase; PAGE, polyacrylamide gel electrophoresis; EGF, epidermal growth factor; WT, wild type; Erk, extracellular signal-regulated kinase; TPA, tetradecanoylphorbol acetate; PKC, protein kinase C; DAG, diacylglycerol.
nucleus. To address this issue, we compared the ability of a panel of receptor mutants to increase the abundance of a group of immediate early genes.

MATERIALS AND METHODS

Cell Culture, Transfection, and CAT Assays—The human epidermoid carcinoma cell line, A431, was a gift from Lynn Heasley (University of Colorado Health Sciences Center). Cells were grown at 37 °C, 7% CO2 in Dulbecco’s modified Eagle’s medium supplemented with 5% fetal bovine serum (Life Technologies, Inc.), 100 units/ml penicillin, 100 μg/ml streptomycin, and 4.5 g/l glucose. Cotransfection experiments were carried out in 6-well plates. The plates onto which 0.5×106 cells were seeded 48 h before. DNA (0.5 μg of pCH110 (Pharmacia Biotech Inc.) + 1.5 μg of AP-1CAT + 2 μg of pBSK (Stratagene)) was applied using 4 ml of LipofectAMINE (Life Technologies, Inc.). The AP-1-CAT reporter contains 10 copies of the SV40 AP-1 binding site upstream of the herpes simplex virus thymidine kinase promoter promoter controlling the CAT gene (11). 5–7 h after transfection, the medium was replaced with medium containing 2% fetal bovine serum. After 12–16 h of recovery, the cells were stimulated with PDGF and incubated for 8–10 h before harvesting.

Cells were lysed by freezing/thawing, and an aliquot of the lysate was used to measure transfection efficiency in a β-galactosidase activity assay. The rest of the lysate was used to measure chloramphenicol acetyltransferase activity (12). After chromatography, the plates were quantitated using a PhosphoImager (Molecular Dynamics).

Construction and Expression of βPDGFR Mutants—The construction of mutant receptors F5, Y740/51, Y771, Y1009, and Y1021 is described in Ref. 6. To construct the Y740 and Y751 mutant receptors, the 1.4-kilobase BamHI-XhoI fragment from RT7 containing F771/1009/121 double mutant (13) was subcloned into the RR3 F751/771 and RR3 F740/771 (14), respectively. The resulting receptor constructs (as a 4.2-kilobase EcoRI-SalI fragment) were subcloned into the pLXSN retroviral expression vector. Amphotrophic virus containing the βPDGFR constructs was produced as described previously (14). A431 cells were infected with the various viruses, and mass populations of drug-resistant cells were selected in 3 ng/ml G418.

Antibodies—The βPDGFR and RasGAP antisera used in these studies were crude polyclonal rabbit antisera and have been previously described (6). The PKB antibody was a generous gift from Dr. A. Toker (Boston Biomedical Research Institute). The anti-SHP-2 antisera was a generous gift from Dr. N. Terrada), a 900-base pair HindIII-XbaI fragment of pKSH321 containing the human glyceraldehyde-3-phosphate dehydrogenase gene (generous gift from Dr. N. Terrada), a 906-base pair PstI fragment of pK (generous gift from Dr. C. Stiles), and a 780-base pair PstI fragment of β-actin (generous gift from Dr. T. Hunter). Probes were labeled with [32P]dCTP (NEN) to a specific activity >5×106 cpm/μg by random priming and used to detect endogenous c-fos, JunB, KC, RhoB, e-myc and glyceraldehyde-3-phosphate dehydrogenase transcripts.

RESULTS AND DISCUSSION

We have previously constructed and characterized a series of βPDGFR phosphorylation site mutants which included F5 (containing tyrosine to phenylalanine substitutions at positions 740, 751, 771, 1009 and 1021), and 4 add-back constructs in which the binding site for one of the receptor-associated proteins was added back to the F5 receptor. These mutants are called Y740/51, Y771, Y1009 and Y1021, and they selectively associate with PI3K, RasGAP, SHP-2 and PLCγ respectively.

When expressed in HepG2 or TRMP cells, we found that the F5 receptor was unable to trigger PDGF-dependent DNA synthesis and that this deficiency could be rescued by restoration of the binding site for PI3K or PLCγ (6). To investigate the degree of redundancy between the signaling pathways initiated by βPDGFR mutants that preferentially engage PI3K or PLCγ, we compared the ability of these receptor mutants to increase expression of a panel of immediate early genes. In addition to the mutants described above, Fig. 1 illustrates two additional mutants that were created for the present study. The Y740 and Y751 constructs contained only one of the two tyrosines required for maximal binding of PI3K.

Initially, we used the TRMP and HepG2 cells expressing these mutants to examine PDGF-dependent changes in the expression of immediate early genes. We found that PDGF or serum induced only marginal changes in the expression of numerous immediate early genes in the parental or βPDGFR-expressing cells (data not shown). Therefore, we introduced the various receptor mutants into A431 cells, a human epidermoid cell line that has no detectable endogenous a or βPDGFRs (19).

Since the receptor had not been expressed in this cell type before, we first characterized the kinase activity and ability of the receptor mutants to associate with several SH2 domain-containing proteins.

Expression Level and Kinase Activity—We introduced the human wild type and mutant βPDGFR constructs into the A431 cells by infection with retroviruses as described under “Materials and Methods.” The infected cells were selected with G418, and the resulting mass populations were used in subsequent experiments. To assess the expression level of the introduced receptor, we subjected total cell lysates to anti-βPDGFR Western blot analysis. As shown in the top panel of Fig. 2A, the empty vector-expressing cells (N2) had no detectable signal, whereas all of the other cell lines expressed the βPDGFR.

Densitometric analysis of the data and standardization with an endogenous protein (p85) showed that the receptor expression level was within 1.5-fold among all of the cell lines (bottom panel of Fig. 2A). Comparison of the relative level of receptor expression in A431 cells with other characterized cell lines (6, 20) indicated that the A431 cells express approximately 1×106 receptors/cell (data not shown).

Next, we looked at the intrinsic kinase activity of the various mutant receptors in vivo and in vitro. Cells were left resting or stimulated with PDGF-BB and lysed, the lysates were normalized for protein concentration and immunoprecipitated with anti-phosphotyrosine antibodies, and the resulting immunoprecipitates were subjected to an anti-PI3K Western blot (Fig. 2B). As expected, the wild type receptor was recovered from PDGF-stimulated but not from unstimulated cells,
whereas the kinase inactive (R634) mutant was not immunoprecipitated even after PDGF stimulation. A comparable amount of all of the other receptor mutants was recovered from PDGF-stimulated cells, indicating that all of the mutants underwent a similar extent of tyrosine phosphorylation. Note that even mutants such as the F5, which is missing 5 phosphorylation sites, is efficiently phosphorylated in response to PDGF. This has been observed in other cell types as well and may reflect the fact that this receptor retains a number of phosphorylation sites, including a major one at Y857.

In addition to examining receptor phosphorylation in vivo, we evaluated the intrinsic kinase activity of the receptors by comparing the ability of the mutants to phosphorylate an exogenous substrate in an in vitro assay. Cells expressing the various mutants were left resting or stimulated with PDGF, the receptor was immunoprecipitated with anti-βPDGFR antibody (30A), and the immunoprecipitates were subjected to an in vitro kinase assay in the presence of an exogenous substrate (glutathione S-transferase-PLCγ). As expected, immunoprecipitates from empty vector or kinase-inactive receptor-expressing cells had no detectable kinase activity (Fig. 2C). In contrast, immunoprecipitates from resting cells expressing the wild type receptor had a low level of kinase activity that was greatly enhanced in samples from PDGF-stimulated cells (Fig. 2C). All of the other mutants had PDGF-stimulated kinase activity that was comparable to the wild type receptor. The modest variation in the kinase activity for the various mutants was not consistently observed. βPDGFR Western blot analysis indicated comparable amounts of receptor in all of the samples (data not shown). These studies demonstrate that the kinase activity of all of the phosphorylation site mutants used in the present study were comparable to that of the wild type receptor.

**Signaling Molecules Recruited by the βPDGFR Mutants**—To determine whether the phosphorylation site mutants selectively associate with a panel of SH2 domain-containing receptor-associated proteins, we performed the following experiments. A431 cells expressing the mutant βPDGFRs were stimulated with PDGF-BB or left resting, the cells were lysed, the receptor was immunoprecipitated, and the samples were resolved by SDS-PAGE and subjected to Western blot analysis using antibodies directed against the receptor or each of the signaling enzymes (Fig. 3A). The receptor Western blot shows that there was a similar amount of receptor in all samples except for the empty vector-expressing cells (N2), which consistently had no detectable receptor. PLCγ, RasGAP, p85, and SHP-2 all coimmunoprecipitated with the wild type receptor provided that it was isolated from PDGF-stimulated cells. In contrast, the R634 receptor coimmunoprecipitated with only trace amounts of these SH2 domain-containing proteins, and exposure to PDGF did not increase their recovery. The F5 receptor bound only very low levels of the signaling enzymes, whereas individually repairing tyrosines 1021, 771, 740/751, or 1009 restored the ability to associate with PLCγ, RasGAP, p85, or SHP-2, respectively. Repairing only one of the two tyrosines of the p85 binding site (the Y740 and Y751 receptors) enabled these receptors to bind more p85 than the F5 construct, and the Y740 receptor consistently associated with p85 better than the Y751 mutant. Since neither the Y740 or the Y751 receptor bound p85 as efficiently as the Y740/51 receptor, we conclude that both 740 and 751 are required for efficient binding of p85 to the βPDGFR. The multiple p85 species that associate with the βPDGFR probably represents multiple p85 isoforms (15), and they are more readily observed in the Y740 and Y751 receptors.
samples, which were resolved on the SDS-PAGE gel longer than the other samples in this figure.

Nishimura et al. (22) have reported that Nck associates with the βPDGFR via a direct interaction, and that Y751 is required for this event. We tested the ability of the various βPDGFR mutants to associate with Nck. Resting or PDGF-stimulated cells were lysed, the lysates were immunoprecipitated with an anti-Nck antisera, the samples were subjected to an in vitro kinase assay in the presence of 0.5 μg of glutathione S-transferase-PLCγ fusion protein. The proteins were resolved on a 10% SDS-PAGE gel, the gel was stained with Coomassie Blue to verify that a comparable amount of exogenous substrate was present in each sample, and then the gel was dried down and subjected to autoradiography. The portion of the resulting autoradiogram containing the fusion protein is presented.

**PDGFR Induces Distinct Pathways Leading to the Nucleus**

**FIG. 2.** Expression and catalytic activity of the various mutant receptors in A431 cells. A, expression levels. Resting A431 cells expressing the various constructs were lysed, and 80 μg of total cell lysate was resolved on a 7.5% SDS-PAGE gel, transferred to Immobilon, and subjected to a βPDGFR Western blot. The lower panel is a densitometric analysis of the Western blot in which the intensity of the receptor band was normalized to an endogenous protein (p85), and the data are expressed as a percentage of WT receptor expression. B, in vivo kinase activity. Cells were stimulated with PDGF-BB (30 ng/ml for 5 min at 37 °C (+) or left unstimulated (−), the cells were lysed, and the lysate was immunoprecipitated with a mixture of anti-phosphotyrosine antisera (PY20/4G10, 1:1). Samples were resolved by SDS-PAGE, transferred to Immobilon, and subjected to Western blot analysis using anti-βPDGFR antisera. The portion of the blot including the approximately 230–140-kDa proteins is shown. C, in vitro kinase activity. The βPDGFR was immunoprecipitated from PDGF-BB stimulated (+) or unstimulated cells (−), and the immunoprecipitates were subjected to an in vitro kinase assay in the presence of 0.5 μg of glutathione S-transferase-PLCγ fusion protein. The proteins were resolved on a 10% SDS-PAGE gel, the gel was stained with Coomassie Blue to verify that a comparable amount of exogenous substrate was present in each sample, and then the gel was dried down and subjected to autoradiography. The portion of the resulting autoradiogram containing the fusion protein is presented.

receptors, including the wild type receptor. At the present time we do not know the functional significance of this observation. These studies indicate that although tyrosine 751 does play a minor role in Nck binding, efficient association of Nck requires that the receptor be phosphorylated at multiple tyrosines in different regions of the receptor (740 and 751 are in the kinase insert, whereas 1009 is in the tail).

Taken together, these results indicate that PLCγ, RasGAP, PI3K, and SHP-2 associate with the βPDGFR in A431 cells much as they do in other cell types and that the binding is dependent on the presence of specific phosphorylation sites. Nck binding, however, appears to be less specific for any particular tyrosine residue as stable binding appears to require tyrosines 1009, 751, and 740.

**Activation of Immediate Early Genes**—To determine the ability of the βPDGFR mutants to activate transcription of immediate early genes, we conducted the following experiments. Cells were arrested by serum deprivation for 48 h then stimulated with EGF (50 ng/ml) or PDGF-BB (10 or 30 ng/ml) for 45 min at 37 °C and lysed, and total RNA was prepared. A time course experiment indicated that c-fos mRNA induction by PDGF-BB or EGF was maximal between 30 and 60 min and returned to the basal level by 2 h (data not shown). Northern blot analysis was performed on 10 μg of total RNA with a panel of 32P-labeled probes. PDGF did not increase the expression of any of the immediate early genes in cells expressing an empty expression vector or the kinase inactive receptor, whereas EGF was able to initiate a robust response in a number of instances (Fig. 4). In cells expressing the wild type receptor, 30 ng/ml PDGF stimulates c-fos to 69% that of the
level induced by EGF, whereas the F5, Y771, and Y1009 receptors gave a very poor response (less than 5% that of the EGF level). The Y1021 receptor triggered two-thirds of the wild type response, whereas the Y740/51 receptor stimulated only one-third of the response obtained with the wild type PDGFR.

Delineation of the importance of the two PI3K binding sites for activation of c-fos was assessed with the Y740 and Y751 receptors. The Y751 but not the Y740 receptor increased c-fos expression; however, the response was much smaller than that observed with the Y740/51 receptor. Thus, both of the tyrosines are required to drive the optimal c-fos response. These studies reveal that engagement of either PI3K or PLCγ leads to c-fos induction; however, there appears to be a quantitative difference between the two pathways.

In addition to this quantitative difference between the PI3K and PLCγ pathways, we detected qualitative differences as additional immediate early genes were examined. JunB was activated by both the wild type and Y1021 receptors, whereas the Y740/51, Y740, or Y751 receptors induced JunB very poorly or not at all. Similarly, KC, a PDGFR-inducible early gene, was modestly activated by the wild type receptor and to a similar extent by the Y1021 receptor, but the Y740/51 receptor failed to induce it. Note that KC is almost insensitive to EGF induction, which is consistent with a previous report (23).

Several additional pieces of information have emerged from these studies. First, none of the immediate early genes were activated by the F5 receptor, which is still capable of binding and activating Src.3 Second, restoring the RasGAP binding site to the F5 receptor did not rescue the ability to increase expression of any of these mRNAs, indicating that RasGAP does not engage any signal relay cascades leading to activation of transcription of these genes. Similarly, restoration of the SHP-2 binding site, which could link the receptor to the Ras pathway via the Grb2/SHP-2 complex, did not activate c-fos, although the Y1009 receptor was able to drive a very small amount of JunB expression. Third, c-myc and rhoB endogenous expression levels are fairly high, and only the WT receptor detectably increased their expression. The fact that none of the mutant receptors tested were potent enough to activate the transcription of these two genes suggests that the simultaneous activation of several pathways may be required for their induction. Finally, the Y740 and Y751 receptors were able to activate rhoB; however, the Y740 receptor was more potent than the Y751 receptor. Nck and PI3K binding to Y740 was not as good as to Y751, suggesting that either rhoB induction requires a reduced amount of binding or some other signaling molecule preferentially associating at site Y740.

3 J. P. Montmayeur and A. Kazlauskas, unpublished observations.
These observations indicate that recruitment of SH2 domain-containing proteins is required for activation of immediate early genes and that the PLCγ and PI3K pathways initiate quantitatively and qualitatively distinct changes in immediate early gene transcription.

Activation of the Ras Pathway Components by the βPDGFR Mutants—The important role of the Ras pathway in mitogenesis and early genes induction (particularly c-fos) prompted us to further investigate the ability of the mutants to activate known components of the Ras signaling cascade. When the PDGFR is expressed in HepG2 or Ph cells, we have been able to measure PDGF-dependent changes in the ratio of GTP/GDP in Ras immunoprecipitates (15, 24). Unfortunately, repeated attempts to measure Ras activation by this direct approach were not successful in the A431 cells (data not shown). Consequently we looked at Ras activation indirectly by examining PDGF-dependent changes of three components of the Ras pathway. First we examined the ability of Grb2 to associate with the βPDGFR mutants. To do so, we immunoprecipitated lysates from resting or PDGF-stimulated cells with an anti-Grb2 antiserum, subjected the immunoprecipitates to an in vitro kinase assay, then dissociated the immune complex and re-immunoprecipitated with a βPDGFR-specific antiserum. The samples were resolved by SDS-PAGE, and the resulting gel was subjected to autoradiography (Fig. 5A). The wild type receptor but not the kinase inactive mutant was able to associate with Grb2 in a PDGF-dependent manner. In comparison with the wild type receptor, F5 bound only very low levels of Grb2, even though tyrosine 716, a previously reported Grb2 binding site (25), is present in the F5 receptor. Restoring the tyrosines at positions 1021, 1009, 771, or 740/751 enabled Grb2 to bind at or above the level that associates with the wild type receptor. A Grb2 Western blot of the Grb2 immunoprecipitates indicated that a similar amount of Grb2 was present in all of the samples (lower panel of Fig. 5A). Thus, binding of Grb2 to the βPDGFR is dependent on the presence of certain tyrosine residues; however, multiple tyrosine residues enable Grb2 binding. It is possible that the apparently low specificity of Grb2 binding is due to the over-expression of the receptor to a high level in these cells. This seems unlikely, however, because more Grb2 associates with the Y1021 receptor, which is expressed at a comparable level as the other receptors (Figs. 2A and 5A). A second explanation for these observations is that Grb2 is not binding to the receptor directly but indirectly, via some other protein(s), as has been shown for both SHP-2 and p85 (24, 26–28). Alternatively, since there is not an optimal Grb2 SH2 domain binding sequence (29) in the βPDGFR, Grb2 binds weakly to nonoptimal sites on the βPDGFR, which is phosphorylated at up to 9 different tyrosine residues in a PDGF-stimulated cell.

As a second approach to elucidate the Ras pathway in βPDGFR signaling, we turned our focus to Shc. We assayed the ability of Shc to coimmunoprecipitate with the βPDGFR and found that Shc bound to the wild type receptor poorly and that none of the phosphorylation site mutants appeared to dramatically alter association with Shc (data not shown). This is consistent with a previous report indicating that mutation of multiple tyrosine residues affected Shc binding to the receptor (30). An alternative approach to evaluate the role of Shc in the βPDGFR pathway was to examine PDGF-dependent tyrosine phosphorylation of Shc. To this end, resting or PDGF-stimulated cells were lysed, and Shc was immunoprecipitated and subjected to anti-Shc Western blot analysis. Phosphorylation of Shc was scored as a retardation in Shc mobility, most easily seen in highest molecular mass Shc isoform. Anti-phosphotyrosine Western blot analysis of the Shc immunoprecipitates indicated that the gel shift assay accurately reflected tyrosine phosphorylation of Shc (data not shown). The wild type but not the kinase inactive receptor stimulated Shc phosphorylation after PDGF stimulation (Fig. 5B). The F5 receptor was unable to mediate robust Shc tyrosine phosphorylation, and restoration of Y771 did not markedly improve this response. In contrast, restoration of the binding sites for PLCγ, SHP-2, PI3K, or even only one of the tyrosine residues of the PI3K binding site enabled the receptor to drive near wild type levels of Shc tyrosine phosphorylation. Given that the kinase activity of all of the receptor mutants was comparable (Fig. 2), it was not immediately obvious why all of the receptors were not able to mediate Shc phosphorylation. Perhaps a weak interaction of Shc with the receptor, which occurs with low specificity, is required for efficient tyrosine phosphorylation of Shc. Finally, these studies show that like Grb2 and Nck, Shc participation in βPDGFR signaling requires that the receptor be tyrosine-phosphorylated; however, there does not appear to be a discernible requirement for phosphorylation of the receptor at a specific tyrosine residue.

A third approach to investigate the Ras pathway was to examine activation of Erks. Cells expressing the various receptor mutants were arrested by serum deprivation and then left...
SHP-2 did not enable the receptor to activate Erk, whereas the WT and Y1021 receptors were able to mediate activation of Erk-1. These observations demonstrate that in A431 there is no direct correlation between the binding of Grb2 or phosphorylation of Shc with Erk activation. Furthermore, the Y740/51 receptor failed to detectably activate Erk but was able to drive a modest increase in c-fos expression (Figs. 4 and 5). Given the well documented link between Erk activation and transcription factors that engage elements within the c-fos promoter, we sought to further characterize the signaling pathways emanating from the Y740/51 and Y1021 receptors.

PI3K Activity and Tetradecanoylphorbol Acetate (TPA) Sensitivity—To verify that the Y740/51 but not the Y1021 receptors engage PI3K activity, anti-phosphotyrosine immunoprecipitates were prepared from resting or PDGF-stimulated cells and subjected to an in vitro PI3K assay. The products of this reaction were resolved by ascending chromatography, and the resulting radioactive phosphatidylinositol 3-phosphate product was quantitated. PI3K activity was best detected in samples that were able to associate with p85, and there was a close correlation between the amount of p85 that bound to the receptor and the amount of PI3K activity present in antiphosphotyrosine immunoprecipitates (compare Figs. 3A and 6A). We consistently found that restoration of the Y740 site resulted in more PI3K activity and more p85 binding than restoration of the Y751 site. These studies show that the Y740/51 receptor but not the Y1021 receptor was able to engage PI3K activity.

The c-fos promoter has numerous well characterized responsive elements, including the TPA-responsive element, which is a downstream target of activated protein kinase C (PKC) family members (32). The PKC family is divided into three groups, one of which is not activated by diacylglycerol (DAG), whereas PKC family members of the other two groups are responsive to DAG (33). Prolonged exposure of cells to TPA, a functional analogue of DAG, down-regulates the PKC isoforms that are activable with DAG but has less of an effect on the DAG-insensitive PKC family members. As a result, it is possible to distinguish the relative contribution of the DAG-sensitive and -insensitive PKC family members by chronic treatment of cells with TPA. This approach was chosen to compare the signaling pathways by which the Y1021 and Y740/51 receptors induced c-fos mRNA expression. Cells expressing the βPDGFR mutants were grown to confluence, arrested by serum deprivation, and then incubated in the presence or absence of 100 ng/ml TPA for 72 h. The cells were then exposed to TPA, PDGF, or vehicle for 30 min, and the RNA was prepared and subjected to Northern blotting using a c-fos probe. Acute stimulation of cells with TPA caused a uniform, robust induction of c-fos in all of the cell types (Fig. 6B). In contrast, only background levels of c-fos were observed in cells chronically exposed to TPA (data not shown). Chronic TPA treatment eliminated the majority of the c-fos induction triggered by PDGF in WT or Y1021 receptor-expressing cells (Fig. 6B). In contrast to the Y1021 receptor, the ability of the Y740/51 receptor to increase the c-fos messenger level was unaffected by chronic exposure to TPA. These findings suggest that the WT receptor activates c-fos primarily through the PLCγ pathway and that this pathway involves a DAG-sensitive PKC family member such as PKCe (34). Furthermore, the PI3K pathway leading to activation of c-fos does not involve a DAG-sensitive enzyme and may instead employ PKCa, as has recently been reported (35). These findings demonstrate that the WT PDGFR activates distinct signaling pathways, each of which is capable of activating nuclear events such as induction of c-fos.

AP-1 Is a Common Nuclear Target for Mutant Receptors—To further compare the ability of the Y740/51 receptors to activate reporter elements within the c-fos promoter we employed an

**FIG. 5.** PDGF-dependent activation of Ras pathway components. A, association with the Grb2 adaptor protein. Quiescent A431 cells expressing βPDGFR mutants were stimulated (30 ng/ml PDGF for 5 min at 37 °C, (+)) or left unstimulated (−). The cells were lysed, the lysates were immunoprecipitated with a monoclonal antibody raised against Grb2, and the samples were subjected to an in vitro kinase assay. The immune complex was dissociated, the proteins were reimmunoprecipitated with an anti-Grb2 antibody, and the samples were subjected to an Erk Western blot. As shown in Fig. 5A, the Y740/51 receptor mutants were stimulated (30 ng/ml PDGF for 5 min at 37 °C, (+)) or left unstimulated (−). The cells were lysed, the lysates were immunoprecipitated with an anti-Shc antibody. The resulting immunoprecipitates were separated on an SDS-PAGE gel and then subjected to an anti-Shc Western blot. The arrow labeled P points to the phosphorylated form of the 68-kDa Shc isoform. The other arrow designates the 48-, 53-, and 68-kDa isoforms. The immunoprecipitates from the Y740 and Y751 receptor mutants were run on a separate gel. C, activation of Erk. Cells were stimulated as described in A, then lysed, and the lysates were immunoprecipitated with an anti-Shc antibody. The resulting immunoprecipitates were separated on an SDS-PAGE gel and then subjected to an anti-Erk Western blot. The position of the phosphorylated and unphosphorylated forms of Erk are indicated by arrows in the right-hand margin. FBS, fetal bovine serum.
Fig. 6. Dissection of the pathways involved in signaling by the Y740/51 and Y1021 receptors. A, PI3K activity in antiphosphotyrosine immunoprecipitates. Cells were grown to confluence, stimulated, and lysed as described in the legend of Fig. 5. The lysates were immunoprecipitated with a mixture of anti-phosphotyrosine monoclonal antibodies (PY20/4G10: 1:1), the resulting immunoprecipitates were subjected to an in vitro PI3K assay, and the phosphatidylinositol 3-phosphate was quantitated by densitometric scanning. Fold activation was calculated as the ratio of radioactivity in phosphatidylinositol 3-phosphate in PDGF-stimulated/unstimulated samples. The results shown are of one experiment representative of three independent assays. B, effects of TPA pretreatment on PDGF-mediated c-fos induction. Cells were made quiescent by serum deprivation and either left resting or incubated with 100 ng/ml TPA for 72 h. The cultures were then left unstimulated (–) or stimulated with 30 ng/ml PDGF-BB (P) or 100 ng/ml TPA (T) for 30 min at 37 °C before harvesting the RNA. C indicates that the cells were preincubated for 72 h in the presence of 100 ng/ml TPA, then further incubated with 30 ng/ml PDGF-BB for 30 min at 37 °C. RNA was purified as described under “Materials and Methods,” and the Northern blot was hybridized with the cDNA probes indicated in the right margin. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

AP-1/CAT reporter construct containing 10 copies of the AP-1 response element (11). The reporter construct was transiently transfected into the cell lines expressing the various βPDGFR mutants, and the cells were arrested in low serum, exposed to PDGF or left unstimulated, then harvested and subjected to a CAT assay. To standardize for transfection efficiency, cells were cotransfected with a LacZ expression vector, and β-galactosidase activity was measured in each cell line after transfection. As shown in Fig. 7, the Y1021, Y740/51, and WT receptors were all able to activate the AP-1 element, whereas R634 and Y751 receptors failed to elicit this response. The F5, Y1009, Y771, and Y740 receptors also initiated a response, however, this response was well below that of the WT receptor, and the variability in replicate experiments made it problematic to determine whether this low level of activation was meaningful (Fig. 7). Since monitoring AP-1 activity focuses only on one of the responsive elements in the c-fos promoter, it is difficult to evaluate the relative contribution of the AP-1-responsive ele-

Fig. 7. AP-1/CAT is preferentially activated by the Y740/51 and Y1021 mutant receptors. Cells were plated and after 8 h transfected with the AP-1/CAT reporter plasmid. After a 12-h incubation, the cells were exposed to 30 ng/ml PDGF-BB or left unstimulated and harvested, and CAT activity was determined. To calculate the relative CAT activity, the samples were corrected for transfection efficiency, and then the CAT activity of PDGF-stimulated samples was divided by the activity recorded in unstimulated samples. The data presented are the mean ± S.D. of three independent experiments. EGF typically gave a 14 ± 3.17-fold induction.

DISCUSSION

We have previously found that a number of proteins associated with the βPDGFR in a highly selective manner, i.e. binding was dependent on phosphorylation of a single or pair of tyrosine residues. This has not been the universal observation for protein-protein interactions involving SH2 domains and tyrosine-phosphorylated proteins. For instance, the ability of PLCγ, p85, RasGAP, or Shc to associate with the EGFR does not depend on phosphorylation of a single tyrosine residue; instead, stable association of these proteins requires phosphorylation of numerous tyrosine residues (36). In the case of the Met/HGF receptor, two adjacent tyrosine residues are required for binding of at least three SH2 domain-containing proteins, indicating that more than one SH2 domain-containing protein uses the same docking site (21). Similar findings have been reported for certain proteins that associate with the βPDGFR, including Grb7 and Shc (5, 30). These studies, together with those presented herein, suggest that SH2 domain-containing proteins associate with the tyrosine-phosphorylated βPDGFR in either a high or low specificity manner. Although the level of receptor expression was quite high in the cells used in these studies, our findings that adaptor proteins associate with the receptor in response to phosphorylation of the receptor at multiple tyrosine residues is similar to the findings of other groups using receptors expressed at a much lower level (5, 30). Consequently, there appears to be multiple mechanisms by which the SH2 domain-containing proteins associate with the βPDGFR. SH2 domain-containing proteins that are adaptor proteins appear to associate via the low specificity route, whereas the SH2 domain-containing proteins that are enzymes or are tightly associated with enzymes interact with the βPDGFR through a high specificity mechanism. This may reflect the need to precisely position the signaling enzymes relative to the membrane/substrate, whereas the adaptor proteins
may serve multiple functions, depending on what else is bound to the receptor as well as the cellular environment. Finally, although it has been reported that in certain cell types the stable association of Nck and Grb2 requires tyrosines 751 and 716, respectively, (22, 25) our data indicate that it is not the case in A431 cells (see Figs. 3B and 5A).

How does the ability to activate a signaling pathway relate to initiation of DNA synthesis? Unfortunately, a relatively small fraction (approximately 10%) of the serum-starved A431 cells expressing the WT receptor entered S phase in response to stimulation with PDGF or serum. Comparison of the mutant receptors indicated that whereas the F5 receptor failed to mediate an increased entry into the S phase, the Y740/51 and Y1021 receptors were as good as the WT receptor in driving PDGF-dependent entry into the cell cycle.4 Because it was only a small fraction of the total population that was entering the S phase, we could not be certain that the changes in immediate early gene expression were occurring in the same cells that were entering the S phase. Consequently, we have not been able to compare the DNA synthesis response with the ability to activate immediate early genes.

Comparison of the Y1021 and Y740/51 receptors for their ability to activate immediate early genes indicated that the response triggered by the Y1021 receptor was much stronger than that seen with the Y740/51 receptor and, furthermore, that the Y1021 receptor response was qualitatively and quantitatively close to that of the WT receptor. These observations suggest that in A431 cells at least some of the intracellular events triggered by the WT βPDGFR predominantly flow through the PLCγ-initiated cascade. In addition, we have found that PLCγ is very robustly activated in A431 cells as compared with HepG2 cells expressing comparable amounts of βPDGFR.5 Thus the relative degree to which signaling pathways are activated by the PDGFR appears to depend on the cell type in which the receptor is expressed. The mechanistic basis of such observations may prove to be quite interesting.

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REFERENCES
1. Yarden, Y., Escobedo, J. A., Kuang, W. J., Yang-Feng, T. L., Daniel, T. O., Tremble, P. M., Chen, E. Y., Ando, M. E., Harkins, R. N., Francke, U., Fried, V. A., Ulrich, A., and Williams, L. T. (1986) Nature 323, 226–232
2. Claesson-Welsh, L. (1994) J. Biol. Chem. 269, 32023–32026
3. Westermark, B. (1990) Acta Endocrinol. 123, 131–142
4. Kazlauskas, A. (1994) Curr. Opin. Genet. Dev. 4, 5–14
5. Yokote, K., Margolis, B., Heldin, C.-H., and Claesson-Welsh, L. (1996) J. Biol. Chem. 271, 30942–30949
6. Valius, M., and Kazlauskas, A. (1993) Cell 72, 321–334
7. Twamlay-Stein, G. M., Pepperkok, R., Ansorge, W., and Courtneidge, S. A. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 7696–7700
8. Barone, M. V., and Courtneidge, S. A. (1995) Nature 378, 509–512
9. Roche, S., Fumagalli, S., and Courtneidge, S. A. (1995) Science 269, 1567–1569
10. Roche, S., McGlade, J., Jones, M., Gish, G. D., Pawson, T., and Courtneidge, S. A. (1996) EMBO J. 15, 4940–4948
11. Aronheim, A., Engelberg, D., Li, N., Al-Alawi, N., Schlessinger, J., and Karin, M. (1994) Cell 78, 949–961
12. Montmayeur, J.-P., and Bierrell, E. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 3135–3139
13. Valius, M., Bazenet, C., and Kazlauskas, A. (1993) Mol. Cell. Biol. 13, 133–143
14. Kazlauskas, A., Kashishian, A., Cooper, J. A., and Valius, M. (1992) Mol. Cell. Biol. 12, 5824–5833
15. Klinghoffer, R. A., Duckworth, B., Valius, M., Cantley, L., and Kazlauskas, A. (1996) Mol. Cell. Biol. 16, 5905–5914
16. Li, W., Pa, H., Skolnik, E. Y., Ulrich, A., and Schlessinger, J. (1992) Mol. Cell. Biol. 12, 2534–2544
17. Kazlauskas, A., Durden, D. L., and Cooper, J. A. (1991) Cell Regul. 2, 413–425
18. Auger, C., and Rougeon, F. (1988) Eur. J. Biochem. 170, 303–314
19. Bravo, R., Barckhardt, J., Curran, T., and Muller, R. (1985) EMBO J. 4, 1103–1107
20. Bazenet, C., and Kazlauskas, A. (1994) Oncogene 9, 517–525
21. Punzetto, C., Bardelli, A., Zhen, Z., Maina, F., dalla Zonca, P., Giordano, S., Graziani, A., Panayotou, G., and Comoglio, P. M. (1994) Cell 77, 261–271
22. Nishimura, R., Li, W., Kashishian, A., Mendlin, A., Zhou, M., Cooper, J., and Schlessinger, J. (1993) Mol. Cell. Biol. 13, 6889–6896
23. Cochran, B. H., Reffel, A. C., and Stiles, C. D. (1983) Cell 33, 939–947
24. Kazlauskas, A. E., Gelderloos, J. A., and Kazlauskas, A. (1996) Mol. Cell. Biol. 16, 6926–6936
25. Arvidsson, A.-N., Rupp, E., Nanberg, E., Downward, J., Runnstrand, L., Wennstrom, S., Schlessinger, J., Heldin, C.-H., and Claesson-Welsh, L. (1994) Mol. Cell. Biol. 14, 6715–6726
26. Bennett, A. M., Yang, T. L., Sugimoto, S., Walsh, C. T., and Neel, B. J. (1996) Proc. Natl. Acad. Sci. U. S. A. 91, 735–739
27. Li, W., Nishimura, R., Kashishian, A., Batzer, A. G., Kim, W. J., Cooper, J. A., and Schlessinger, J. (1994) Mol. Cell. Biol. 14, 509–517
28. Wang, J., Auger, K. R., Jarvis, L., Shi, Y., and Roberts, T. M. (1995) J. Biol. Chem. 270, 12774–12780
29. Songyang, Z., Shoelson, S. E., Chaudhuri, M., Gish, G., Pawson, T., Haer, W. G., King, F., Roberts, T., Ratnoff, S., Leshleider, R. J., Neel, B. J., Birge, R. B., Fajardo, J., Chou, M. M., Hanafusa, H., Schaffhausen, B., and Cantley, L. C. (1993) Cell 72, 767–778
30. Yokote, K., Morii, S., Hansen, K., McGlade, J., Pawson, T., Heldin, C.-H., and Claesson-Welsh, L. (1994) J. Biol. Chem. 269, 15337–15343
31. Vaillancourt, R. R., Heasley, L. E., Zamarripa, J., Storey, B., Valius, M., Kazlauskas, A., and Johnson, G. L. (1995) Mol. Cell. Biol. 15, 3644–3553
32. Verma, I. M., and Sassone-Corsi, P. (1987) Cell 47, 513–514
33. Nishizuka, Y. (1995) FASEB J. 9, 484–496
34. Moriya, S., Kazlauskas, A., Akimoto, K., Hirai, S., Mizuno, K., Takenawa, T., Fukui, Y., Watanabe, Y., Ozaki, S., and Ohno, S. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 151–155
35. Akimoto, K., Takahashi, R., Moriya, S., Nishikawa, N., Takayanagi, J., Kimura, K., Fukui, Y., Osada, S.-I., Mizuno, K., Hirai, S.-I., Kazlauskas, A., and Ohno, S. (1996) EMBO J. 15, 788–797
36. Soler, C., Beguinot, L., and Carpenter, G. (1994) J. Biol. Chem. 269, 12320–12324

4 M. Valius and Z. Krivickiene, unpublished observations.
5 M. Valius and A. Kazlauskas, unpublished observations.