Differences in Substrate Specificities of α and β Platelet-derived Growth Factor (PDGF) Receptors

CORRELATION WITH THEIR ABILITY TO MEDIATE PDGF TRANSFORMING FUNCTIONS*

(Received for publication, September 3, 1992, and in revised form, January 8, 1993)

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Recombinant expression of either the α or β platelet-derived growth factor (PDGF) receptors in 2D hematopoietic cells allows efficient coupling of PDGF with mitogenic and chemotactic signaling pathways inherently expressed by those cells. PDGF-BB stimulation of 32D-αR or βR cells results in anti-P-Tyr recovery of cellular proteins possessing similar as well as distinct phosphotyrosine signals. Comparison of the ability of each receptor to couple with known second messengers revealed that both receptors associated with tyrosine phosphorylated phospholipase C-γ (PLCγ) and phosphatidylinositol 3-kinase (p85) with similar stoichiometry. However, the β platelet-derived growth factor receptor (PDGFR) was significantly more efficient at in vivo tyrosine phosphorylation of GTPase-activating protein (GAP). Similar differences in binding affinity for GAP were observed in NIH/3T3 cells which express both receptors. To quantitate the affinities of each receptor for GAP or PLCγ, we utilized baculovirus-expressed α and β PDGFRs purified by anti-P-Tyr affinity chromatography. Exposure of immunobots containing bacterially expressed GAP or PLCγ to activated α or β PDGFR led to a comparable high affinity binding of each receptor to PLCγ, while the β PDGFR showed a 5-fold higher binding affinity for GAP.

In an effort to correlate differences in their substrate specificities with biological properties of the receptors, we compare their abilities to enhance PDGF-A transforming function in NIH/3T3 cells. Cotransfection of PDGF-A with the α PDGFR increased PDGF-A transforming activity by ~2-fold. However, cotransfection with a chimeric receptor with the catalytic domain of the β PDGFR but possessing α PDGFR ligand binding properties resulted in 17-fold enhancement of PDGF-A transformation. These findings argue that differences in α and β PDGFR receptor substrate specificity in NIH/3T3 fibroblasts correlate with greater transforming activity mediated by the β PDGFR.

Platelet-derived growth factor (PDGF)* is a potent mitogen for connective tissue cells (1, 2). This growth factor is comprised of dimers of A and B chains encoded by distinct genes (3, 4). All three PDGF isoforms (PDGF-AA, PDGF-BB, and PDGF-AB) have been identified (5–7). Moreover, they bind with different affinities to two related receptor molecules, designated α and β PDGFRs which are also encoded by distinct genes (8, 9). Accumulating evidence indicates that PDGF-induced receptor activation involves recruitment of receptor dimers (10–13). In fibroblasts where both PDGFRs are expressed, PDGF-BB activates αβ, ββ, and αα receptor dimers. In contrast, PDGF-AA induces formation of only αα receptor homodimers, while PDGF-AB is capable of recruiting αα as well as αβ dimers (14).

Several PDGF substrates have recently been identified. These include phospholipase C-γ (PLCγ) (15), GTPase-activating protein (GAP) (16, 17), and the 85 kDa subunit of the phosphatidylinositol 3-kinase (18). Each has been shown to undergo rapid tyrosine phosphorylation and/or physical association with PDGFRs in response to PDGF triggering (15–19). PLCγ hydrolyzes phosphatidylinositol 4,5-bisphosphate into two second messengers, 1,2-diacylglycerol and inositol 1,4,5-trisphosphate. The former activates protein kinase C, and the latter promotes the release of Ca²⁺ from intracellular stores (20). GAP enhances hydrolysis of ras-GTP to ras-GDP which normally inactivates ras function, but GAP may also be involved in Ras effector function (21, 22). A phosphatidylinositol-3 kinase was initially identified in immunocomplexes with the v-src protein and later found to be physically associated with a variety of tyrosine kinases including PDGFRs (23–26). Phosphatidylinositol 3-kinase phosphorylates the inositol ring of phosphatidylinositol at the D3 position, but there is yet no clue as to the biological functions of such metabolites (27, 28).

The ability of PDGFRs to associate with and/or tyrosine phosphorylate known substrates has generally been investigated utilizing fibroblasts expressing both receptors. Thus, it has been difficult to quantitatively compare the ability of each receptor to interact with specific substrates. We have previously described 32D hematopoietic cell lines engineered to independently express equivalent levels of α or β PDGFRs. We showed that ligand-induced stimulation of either receptor in this cell system leads to quantitatively similar levels of calcium mobilization, PI turnover, chemotaxis, and mitogenic responses (29). Our present studies were undertaken in an effort to compare the ability of each receptor independently to interact with known intracellular signaling molecules in vivo as well as in vitro.

MATERIALS AND METHODS

Transfection Analysis—The interleukin-3-dependent mouse hematopoietic cell line 32D has been described previously (30). DNA
transfection of 32D cells was performed by electroporation (31). Mass populations of stably transfected cells were selected by their ability to survive in growth medium containing mycophenolic acid (32). Transfection of NIH/3T3 fibroblasts was performed with the calcium-phosphate precipitation method (33) using 40 pg of calf thymus DNA as a template and a serial dilution of transfected NIH/3T3 cells were plated for 14-21 days. Depending upon the DNA construct, NIH/3T3 transfectants were selected for survival in growth medium containing mycophenolic acid (32) or geneticin (750 µg/ml).

**Immunoblot and Immunoprecipitation Analysis**—32D cells were washed twice in Dulbecco’s modified Eagle medium and incubated at 37 °C for 2 h in serum-free medium. The quiescent 32D cells were then stimulated with PDGF-BB (Upstate Biotechnology, Inc.) (100 ng/ml) for 5 min at 37 °C. For immunoprecipitation analysis, stimulated cells were treated with 5 mM diisopropyl fluorophosphate (DFP) at 4 °C for 5 min, and lysed in a P-Tyr buffer containing 50 mM HEPES, pH 7.5, 1% Triton X-100, 50 mM NaCl, 50 mM NaF, 10 mM sodium pyrophosphate, 5 mM EDTA, 1 mM Na3 VO4, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml aprotinin, 10 µg/ml leupeptin, and 5 mM DFP. Soluble lysates (2 mg) were immunoprecipitated with anti-P-Tyr antibody (Upstate Biotechnology, Inc.). Immunoprecipitates were resolved on SDS-PAGE and immunoblotted with anti-P-Tyr, anti-PLCy (Upstate Biotechnology, Inc.), anti-p85 (Upstate Biotechnology, Inc.), or anti-p55 (Upstate Biotechnology, Inc.), or anti-p56 (34).

**Receptor-associated Phosphatidylinositol 3-Kinase Assays**—For measurement of in vivo PDGF-associated phosphatidylinositol 3-kinase activity, quiescent 32D transfectants were exposed to PDGF-BB (100 ng/ml) for 5 min at 37 °C, incubated with 5 mM DFP at 4 °C for 5 min, and lysed in a calcium-free buffer containing 20 mM Tris (pH 8), 137 mM NaCl, 2.7 mM KCl, 1 mM MgCl2, 1 mM CaCl2, 1% Nonidet P-40, 10% glycerol, 1 mM Na3 VO4, 5 mM DFP, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml aprotinin and leupeptin. Soluble lysates (2 mg) were immunoprecipitated with anti-P-Tyr antibody (Upstate Biotechnology, Inc.). Immunoprecipitates were recovered with protein A-Sepharose and assayed for phosphatidylinositol 3-kinase activity by the method of Scatchard (39).

**RESULTS**

**Patterns of Tyrosine-phosphorylated Proteins in Vivo**

**Suggest Differences in Substrate Specificities of α and β PDGF Receptors**—We have previously established 32D cell lines which express α or β PDGF receptors at similar levels (41). These cell lines enabled us to quantitatively compare the pattern of tyrosine-phosphorylated proteins stimulated by PDGF-BB in the same target cell. Accordingly, 32D-αR and βR cells were each challenged with saturating levels of PDGF-BB which interacts with both receptors with similar affinity (41). Following exposure for 5 min at 37 °C, cells were lysed and immunoprecipitated with anti-P-Tyr. Immune complexes enriched for phosphotyrosine-containing proteins were then subjected to immunoblot analysis using anti-P-Tyr.

As shown in Fig. 1, PDGF-BB stimulation of 32D-αR or βR cells resulted in anti-P-Tyr recovery of equivalent levels of each PDGF subtype as determined by the signal intensity of bands at ~200 kDa for the β PDGF and 190 kDa for the α PDGF, respectively. Furthermore, activation of either the

![Fig. 1. In vivo PDGF-induced tyrosine phosphorylation in 32D transfectants. Quiescent 32D cells were treated (+) or untreated (-) with PDGF-BB (100 ng/ml) for 5 min at 37 °C. 32D-αR (lanes 1 and 2) and 32D-βR (lanes 3 and 4) were then lysed, and soluble fractions (2 mg) were immunoprecipitated with anti-P-Tyr. Immunoprecipitates were electrophoretically separated, transferred to immobilon-P, and blotted with anti-P-Tyr antibody.](image-url)
α PDGFR or β PDGFR resulted in anti-P-Tyr recovery of a set of phosphotyrosine-containing proteins of similar respective sizes including p140, p85, p75, p70, and p65 (Fig. 1). However, we also observed a distinct subset of proteins (p120, p95, and p57) which were more efficiently tyrosine phosphorylated by β PDGFR. In contrast, the α PDGFR demonstrated more efficient tyrosine phosphorylation of proteins with estimated molecular masses of 48 and 50 kDa, respectively. These results were reproducible in several independent experiments, suggesting that the α and β PDGFR kinases may have different specificities for certain signaling molecules in vivo.

Comparison of in Vivo Interactions of α and β PDGFR with Known Substrates—By use of 32D-αR and βR cell lines, it was possible to quantitatively compare the ability of α and β PDGFRs to undergo ligand-induced association with and/or tyrosine phosphorylation of specific PDGFR substrates. For these studies, cells were either untreated or exposed to PDGF-BB, and total lysates were subjected to immunoprecipitation using anti-P-Tyr. The immune complexes were then immunoblotted with anti-P-Tyr or antisera directed against specific substrates. As shown in Fig. 2, PDGF-BB stimulation of the two cell lines resulted in anti-P-Tyr recovery of equivalent amounts of α or β PDGFRs (panel A) and of phosphatidylinositol 3-kinase p85 subunit (panel B). To further compare the ability of either receptor to undergo ligand-induced association with the phosphatidylinositol-3 kinase, anti-P-Tyr recoverable proteins were analyzed for phosphatidylinositol 3-kinase activity. As shown in Fig. 2C, PDGF-BB stimulated anti-P-Tyr recovery of similar levels of phosphatidylinositol 3-kinase activity in 32D-αR or -βR, respectively.

We next compared the ability of each receptor to interact in vivo with two other well characterized PDGFR substrates, PLCγ (15) and GAP (16, 17). Immune complexes enriched for phosphotyrosine-containing proteins were subjected to immunoblot analysis using anti-P-Tyr, anti-PLCγ, or anti-GAP antibodies. The results in Fig. 3A demonstrate the presence of activated α PDGFRs, whose level was estimated to be 1.9-fold higher than that observed for activated β PDGFRs, based upon anti-P-Tyr signal intensities. At the same time, tyrosine phosphorylation of PLCγ was estimated to be 1.3-fold higher in 32D-αR than that observed in 32D-βR. When normalized, the α PDGFR was around 70% as efficient as the β PDGFR at PLCγ tyrosine phosphorylation. These results are consistent with previous findings indicating that PDGF-BB stimulation induces similar levels of PI hydrolysis in 32D-αR or βR cells (29). In striking contrast, GAP appeared to be a much better in vivo substrate of the β PDGFR as compared to α PDGFR under the same assay conditions (Fig. 3C). Similar results were obtained with at least three independently derived mass cultures of 32D cells expressing either α or β PDGFR. The respective levels of immunoreactive GAP, PLCγ, and phosphatidylinositol 3-kinase p85 in total cell lysates from 32D-αR were similar to those in 32D-βR (data not shown).

Comparison of in Vivo Interaction of α and β PDGFRs with GAP—To further explore the mechanism underlying the lower stoichiometry of ligand-induced GAP association and/or tyrosine phosphorylation by the α PDGFR, we compared the abilities of activated α and β PDGFRs to interact with GAP in NIH/3T3 fibroblasts where both PDGFRs are normally expressed. Accordingly, confluent NIH/3T3 cells were rendered quiescent by incubation overnight in serum-free medium and then exposed to a saturating concentration of PDGF-AA or PDGF-BB for 5 min, after which cell lysates were subjected to immunoblot analysis with anti-P-Tyr antibody. As shown in Fig. 4A, addition of either homodimer led to tyrosine phosphorylation of a similar level of PDGFRs. The same cell lysates (2 mg) were also immunoprecipitated with anti-GAP and immune complexes subjected to immunoblot analysis using anti-P-Tyr or anti-GAP.

As shown in Fig. 4B, PDGF-AA and PDGF-BB both stimulated equivalent levels of anti-GAP recoverable p64/62 (42). However, PDGF-AA induced a 9-fold lower level of tyrosine-phosphorylated GAP and anti-GAP recoverable PDGFRs as compared to PDGF-BB. Fig. 4C, shows that similar levels of

![Fig. 2. Comparison of anti-P-Tyr recoverable p85 and phosphatidylinositol 3-kinase activities in PDGF-stimulated 32D transfecants.](image_url)

![Fig. 3. Comparison of PDGF-BB-induced tyrosine phosphorylation of PLCγ and GAP by either α or β type PDGFRs.](image_url)
GAP were immunoprecipitated by anti-GAP antiserum in cells stimulated by PDGF-AA or PDGF-BB. Since differences observed in the level of anti-GAP recoverable PDGFRs could not be attributed to differences in levels of activated PDGFRs (see Fig. 4A), our results imply that the decreased level of GAP tyrosine phosphorylation by activated α PDGFRs reflects its lower affinity for GAP.

To further establish that differences observed in GAP tyrosine phosphorylation did not reflect differences in PDGF binding specificities of the two PDGFRs, we examined the ability of PDGF-AA to stimulate GAP tyrosine phosphorylation in cells engineered to express a similar level of either the α PDGFR or a chimeric PDGFR. The latter, designated the α\(^{\beta\delta\varepsilon\gamma}\), comprises a β PDGFR, in which the first 3 IgG-like domains were substituted by the homologous domain of the α PDGFR (9), conferring α PDGFR binding properties (41). Accordingly, NIH/3T3 cells were transfected with vector alone (NIH), α PDGFR (NIH-α\(^{\beta\delta\varepsilon\gamma}\)), or α\(^{\beta\delta\varepsilon\gamma}\) (NIH-α\(^{\beta\delta\varepsilon\gamma}\)), marker selected, and subjected to binding studies using \(^{125I}\)-PDGF-AA. As shown in Fig. 5, B and C, both NIH-α\(^{\beta\delta\varepsilon\gamma}\) and NIH-α\(^{\beta\delta\varepsilon\gamma}\) cell lines exhibited similar levels of PDGF-AA-binding sites/cell. Each showed 1.5-fold more sites than observed in the parental NIH cells, which expressed 6.5 × 10^3 sites/cell (see Fig. 5A).

To examine the ability of PDGF-AA to stimulate tyrosine phosphorylation of GAP in each cell line, cultures were exposed to saturating concentrations of PDGF-AA or -BB. Total cell lysates (2 mg) were then immunoprecipitated with anti-P-Tyr and the immune complexes subjected to immunoblot analysis using anti-P-Tyr (panel D), anti-PLCγ (panel E), or anti-GAP (panel F). The results in Fig. 5D indicate that PDGF-AA induced equivalent levels of activated PDGFRs in NIH-α\(^{\beta\delta\varepsilon\gamma}\)R or NIH-α\(^{\beta\delta\varepsilon\gamma}\)R2R, which were ~50% higher than that observed in NIH cells. Under these conditions, PDGF-AA stimulated anti-P-Tyr recovery of PLCγ with similar relative stoichiometry (see Fig. 5E). In contrast, the level of PDGF-AA-stimulated GAP tyrosine phosphorylation in NIH-α\(^{\beta\delta\varepsilon\gamma}\)R was ~6-fold higher than that observed in NIH-α\(^{\beta\delta\varepsilon\gamma}\)R and 9-fold greater than observed with the parental NIH cells (Fig. 5F). These results strongly argue that the catalytic domain of β PDGFR exhibits significantly greater ability than the α PDGFR to induce GAP tyrosine phosphorylation and receptor association.

Comparison of Transforming Activity of PDGF-AA Coexpressed with Exogenous α or α β PDGFRs—We have previously demonstrated that PDGF-BB is 10–100-fold more efficient than PDGF-AA at inducing transformation of NIH/3T3 cells (43). It is known that PDGF-BB stimulates α as well as β PDGFRs (41). Thus, the greater transforming efficiency of PDGF-BB could be due to a quantitative increase in the level of activated receptors, differences in α and β PDGFR substrate specificity, or both. To investigate these possibilities, we utilized the αβ chimeric PDGFR (α\(^{\beta\delta\varepsilon\gamma}\)) and compared transforming activity of PDGF-A cotransfected with either wild type α PDGFR or the chimeric PDGFR. As shown in Fig. 6 and Table I, transfection with PDGF-A resulted in ~2.5 × 10^9 transformed foci/picromole of DNA (panel 6B), while cotransfection of PDGF-A with the wild type α PDGFR resulted in a 2-fold higher level of focus formation as compared to that observed with PDGF-A alone (panel 6E). Cotransfection with the chimeric α β PDGFR (α\(^{\beta\delta\varepsilon\gamma}\)) led to a greater than 17-fold increase in PDGF-A transforming efficiency (panel 6F). These results indicate the β PDGFR catalytic domain is more efficient in mediating PDGF transforming function due to differences in substrate specificity of α and β PDGFRs.

In Vitro Affinity for GAP Is Significantly Lower for α Compared to β PDGFRs—In an effort to directly compare their affinities for GAP, we examined the ability of either tyrosine phosphorylated α or β PDGFR expressed in the baculovirus system to associate with bacterially expressed recombinant GAP or PLCγ immobilized on a solid matrix. Accordingly, the lgt11 was engineered to express the fusion protein composed of coding region of either GAP (lgt11-GAP) or PLCγ (lgt11-PLCγ) fused to the β-galactosidase (β-gal) gene. Bacteria infected with recombinant bacteriophage were then induced to express β-gal, β-gal fused to PLCγ (β-gal-PLCγ) or GAP (β-gal-GAP), respectively. Proteins in lysates were then subjected to SDS-PAGE analysis and transferred to nitrocellulose. Filters were then stained with Amido Black or immunoblotted with antibodies specific to β-gal, GAP, or PLCγ.

As shown in Fig. 7A, Amido Black staining confirmed the expression of similar levels of recombinant protein in each of the bacterial lysates. Fig. 7B shows that the majority of proteins whose expression was specifically induced in bacteria infected by lgt11-GAP or lgt11-PLCγ showed specific reactivity with anti-β-gal antibody. This antibody also recognized a major species of 116 kDa, consistent with the size of the β-gal protein itself, in total lysate prepared from bacteria in-
**α and β PDGFR Substrate Specificity**

**Fig. 5.** Scatchard analysis of PDGF-A binding to NIH, NIH-α³⁴０R, and NIH-α³⁴₀β³⁴₂R transfectants and comparison of PDGF-A-induced anti-P-Tyr recovery of PLCγ and GAP in these cell lines. Panels A-C, NIH/3T3 transfected with vector alone (NIH) (panel A), αR (NIH-α³⁴₀R) (panel B) and α³⁴₀β³⁴₂R (NIH-α³⁴₀β³⁴₂R) (panel C) were subjected to binding analysis using labeled PDGF-A as described under “Materials and Methods.” The binding data were then analyzed by the method of Scatchard (39). Panels D-F, Quiescent NIH/3T3 transfectants were untreated (lanes 1-3) or exposed to PDGF-AA (100 ng/ml) (lanes 4-6) or PDGF-BB (100 ng/ml) (lanes 7-9) for 5 min at 37°C. Cells were then lysed and soluble fractions (2 mg) were immunoprecipitated with anti-P-Tyr. Immunoprecipitates were electrophoretically separated, transferred to immobilon-P, and blotted with anti-P-Tyr (panel D), anti-PLCγ (panel E), or anti-GAP (panel F).

**Fig. 6.** Comparison of transforming activity of LTR-2 (A), PDGF-A (B), PDGF-B (C), PDGF-A/LTR-2 (D), PDGF-A/αPDGFR (E), and PDGF-A/α³⁴₀β³⁴₂R (F). NIH/3T3 cells were transfected with 1 μg of each construct by means of calcium-phosphate precipitation technique (33). Plates were stained with Giemsa at 14 days after transfection.

fected by λgt11 alone. When identical filters were immunoprobed with anti-GAP peptide serum, a heterologous series of proteins with a molecular mass ranging from 120–190 kDa was specifically detected in lysate of λgt11-GAP-infected bacteria (Fig. 7C). Moreover, immunodetection of these proteins was specifically blocked by preincubation of the antisera with the homologous GAP peptides (Fig. 7D). In contrast, the anti-PLCγ serum detected a predominant single band with molecular mass of ~210 kDa specific to λgt11-PLCγ bacterial lysates (Fig. 7E). These results demonstrated specific expression of β-gal-GAP and β-gal-PLCγ fusion proteins in each bacteria.

The specific binding of α or β PDGFRs to GAP, phosphatidylinositol 3-kinase or PLCγ has been shown to be dependent on specific tyrosine phosphorylation of these receptor molecules. Thus, to compare their in vitro association with bacterially expressed GAP or PLCγ, we utilized recombinant PDGFR molecules produced in SF9 insect cells. At high levels of expression in this system, PDGFR molecules became tyrosine phosphorylated, allowing their enrichment by means of anti-P-Tyr immunoadfinity chromatography (36). Accordingly, lysates of bacterially expressed β-gal, β-gal-GAP, or β-gal-PLCγ proteins were subjected to SDS-PAGE, and immunoblots were incubated in the presence of saturating concentrations of baculovirus α PDGFRs. Filters were then washed extensively and immunoblotted with either anti-α PDGFR monoclonal antibody (Fig. 8A) or anti-P-Tyr (Fig. 8B). Fig. 8 shows that the α PDGFR bound to GAP and
PLCγ specifically, since both anti-P-Tyr and anti-α PDGFR antibodies detected proteins with a similar pattern to those observed with anti-GAP or anti-PLCγ antibodies (see Fig. 7, C and E). In the absence of PDGFR preincubation, neither anti-α PDGFR or anti-P-Tyr antibodies showed evidence of nonspecific binding to β-gal or β-gal-GAP or β-gal-PLCγ proteins (data not shown).

We next sought to quantitatively compare the abilities of α and β PDGFRs to associate with GAP and PLCγ. Accordingly, lysates of bacterially expressed β-gal-PLCγ and β-gal-GAP were subjected to immunoblot analysis using increasing concentrations of baculovirus expressed β-galactosidase. After extensive washing, specifically absorbed PDGFRs were immunodetected with anti-P-Tyr since this antibody detected β-specific, since both anti-P-Tyr and anti-α PDGFR antibodies showed evidence of nonspecific binding to β-gal or β-gal-GAP or β-gal-PLCγ antibodies (see Fig. 7, C and E). In the absence of PDGFR preincubation, neither anti-α PDGFR or anti-P-Tyr antibodies showed evidence of nonspecific binding to β-gal or β-gal-GAP or β-gal-PLCγ proteins (data not shown).

| TABLE I
Comparison of transforming activity of PDGF-A coexpressed with α or αββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββββbeta construct

Transfection was performed with calf thymus DNA (40 μg) as carrier and serial dilution of indicated plasmids by the calcium-phosphate precipitation method. Focus formation was scored at 14–21 days and transforming efficiency was calculated in focus forming units relative to that induced by PDGF-A cotransfected with LTR-2 expression vector. By comparing activities of the two receptors with respect to several known PDGFR substrates, we also demonstrated similarities as well as striking differences in their ability to interact with specific target molecules. In vitro, interactions of receptor kinases with some known substrates is dependent upon phosphorylation of specific tyrosine residues within the receptor kinase. In addition, these interactions are dependent upon the presence of particular regions within the substrates, designated src homology 2 (SH2) domains. The most well characterized interactions of receptor kinases with their substrates involve those with the phosphatidylinositol 3-kinase p85 regulatory subunit. Accumulating evidence indicates that phosphorylation of 2 different tyrosine residues within the respective kinase insert domains of α and β PDGFRs is required for this interaction.

DISCUSSION

Our present studies demonstrate differences in substrate specificities of α and β PDGFRs as indicated by their distinct patterns of PDGFR-activated anti-P-Tyr recoverable phosphoproteins in a common target cell. These results are consistent with findings of Eriksson et al. using a different cell system. By comparing activities of the two receptors with respect to several known PDGFR substrates, we also demonstrated similarities as well as striking differences in their abilities to interact with specific target molecules in vivo and in vitro.

Interactions of receptor kinases with some known substrates is dependent upon phosphorylation of specific tyrosine residues within the receptor kinase. In addition, these interactions are dependent upon the presence of particular regions within the substrates, designated src homology 2 (SH2) domains. The most well characterized interactions of receptor kinases with their substrates involve those with the phosphatidylinositol 3-kinase p85 regulatory subunit. Accumulating evidence indicates that phosphorylation of 2 different tyrosine residues within the respective kinase insert domains of α and β PDGFRs is required for this interaction.
within the tyrosine kinase receptors, which interact with phosphatidylinositol 3-kinase p85 subunit (50). Our present studies as well as those of several laboratories indicate that both PDGFRs have similar affinities for this substrate. Previous reports have shown that activation of α or β PDGFRs independently expressed in 32D hematopoietic cells results in similar levels of PI hydrolysis and calcium mobilization due to PLCγ activation (29). While the specific sites not yet been identified, receptor tyrosine phosphorylation (35, 45, 47-49). These sites at tyrosine residues 731 and 742 appear to be required (15). Our present findings demonstrate similar efficiency of PLCγ tyrosine phosphorylation by the two receptors in vivo, as well as comparable affinities for their in vitro interaction with PLCγ. Thus, the abilities of these related receptors to interact with certain substrates appear to be very similar.

In striking contrast to our findings with phosphatidylinositol 3-kinase and PLCγ, we observed marked differences in the ability of the α and β PDGFRs to associate with and tyrosine phosphorylate GAP in vivo. In addition, we demonstrated around 5-fold lower affinity of α PDGFR interaction with GAP both in vivo and in vitro. These findings suggest that differences observed in α and β PDGFR tyrosine phosphorylation of GAP may not be limited to differences in the extent of GAP tyrosine phosphorylation (i.e. lower affinity of activated α PDGFR for GAP), but also may relate to differences in preferred sites of GAP tyrosine phosphorylation.

Recently, two independent laboratories have reported that the ability of the β PDGFR to associate with GAP was determined by phosphorylation of tyrosine 771 located within the carboxyl half of its kinase insert domain (45, 49). Sequence comparison between the two PDGFRs indicates that this tyrosine is conserved within the α PDGFR. However, further inspection of this alignment reveals that amino acid residues surrounding tyrosine 771 within the β PDGFR (homologous to tyrosine 762 in the α PDGFR) has significantly diverged between the two receptors. Thus, a more detailed molecular genetic analysis of kinase insert domains of the α PDGFR may help in determining whether or not sequence differences between the receptors in this region could contribute to a lower affinity between GAP and the α PDGFR.

In 32D hematopoietic cells, activation of either independently expressed PDGFR results in similar mitogenic and chemotactic responses (29). Thus, the differences observed in GAP phosphorylation do not directly correlate with either of these biological effects. The β PDGFR is very efficient at phosphorylating GAP (16). Thus, it is not possible to exclude that a threshold requirement is met by the α PDGFR despite its lower affinity GAP interaction. A number of other receptor kinases exhibit low affinity interactions with GAP, PLCγ, or both and yet are capable of stimulating good mitogenic re-

Fig. 8. In vitro association and/or tyrosine phosphorylation of bacterially expressed GAP and PLCγ by α PDGFR. Bacteria expressing β-gal (lane 1), β-gal-PLCγ (lane 2), or β-gal-GAP (lane 3) proteins were lysed. Lysates (100 µg/lane) were resolved by SDS-PAGE and transferred to immobilon-P. The transferred blots were then incubated in the presence of 1.88 nM concentration of α PDGFR expressed in SF9 cells. Filters were then immunoblotted using anti-αPDGFR (panel A) or anti-P-Tyr (panel B) antibodies.

Fig. 9. Comparison of in vitro association and/or tyrosine phosphorylation of PLCγ and GAP by purified α or β type PDGFRs. Bacteria expressing either β-gal-PLCγ (panels A and B) or β-gal-GAP (panels C and D) proteins were lysed. Lysates (100 µg/lane) were resolved by SDS-PAGE and transferred to immobilon-P. The transferred blots were then incubated in the presence of indicated concentrations of purified α PDGFR (panels A and C) or β PDGFR (panels B and D) expressed in SF9 cells. Filters were then immunoblotted using anti-P-Tyr.
influence the biological responses mediated by these related cells. Recently, Eriksson et al. (44) reported differences in the ability of α and β PDGFRs to induce actin reorganization in porcine aortic endothelial cell lines. All of these findings support the concept that PDGF signaling is determined not only by the levels of α and β PDGFR expression, but also by their respective interactions with distinct substrates in different cell types. The PDGF ligand/receptor system is further complicated by the existence of three PDGF isoforms which can induce formation of different combinations of activated receptor dimers (14). Thus, it is likely that the PDGF isoforms present in a particular microenvironment as well as relative levels of expression of the two receptors influence the biological responses mediated by these related receptors.

Acknowledgments—We thank J. S. Gutkind and C. J. Molloy for helpful discussions. The expert technical assistance of C. Kniesley, J. Artrip, and M. May-Siroff is also acknowledged.

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FIG. 10. Scatchard analysis of PDGFR association and/or tyrosine phosphorylation of recombinant PLCγ or GAP in vitro. Denstometric analysis was performed on anti-P-Tyr signals shown in Fig. 9. Following standardization with anti-P-Tyr signals derived from known concentrations of purified α or β PDGFRs, the data were then analyzed by the method of Scatchard (39).
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