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Independent expression of human α or β platelet-derived growth factor receptor cDNAs in a naive hematopoietic cell leads to functional coupling with mitogenic and chemotactic signaling pathways

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ABSTRACT **Distinct genes encode α and β platelet-derived growth factor (PDGF) receptors that differ in their abilities to be triggered by three dimeric forms of the PDGF molecule. We show that PDGF-receptor mitogenic function can be reconstituted in a naive hematopoietic cell line by introduction of expression vectors for either α or β PDGF receptor cDNAs. Thus, each receptor is independently capable of coupling with mitogenic signal-transduction pathways inherently present in these cells. Activation of either receptor also resulted in chemotaxis, alterations in inositol lipid metabolism, and mobilization of intracellular Ca^{2+}. The magnitude of these functional responses correlated well with the binding properties of the different PDGF isoforms to each receptor. Thus, availability of specific PDGF isoforms and relative expression of each PDGF-receptor gene product are major determinants of the spectrum of known PDGF responses.**

Platelet-derived growth factor (PDGF) is a major connective tissue cell mitogen that is thought to play an important role in normal wound healing. The gene for the PDGF B chain has been shown to be the human homologue of the v-*sis* oncogene (1–3), and the abnormal expression of PDGF has been implicated in a variety of histopathologic conditions, including arteriosclerosis and fibrotic diseases as well as cancer (4). The major form of PDGF isolated from human platelets is believed to be an AB heterodimer (5), but there is evidence for the natural occurrence of all three dimeric forms of the *PDGFA* and *PDGFB* gene products (6, 7).

Recently, our group identified and cloned the cDNA of another human PDGF receptor (PDGFR), designated the α PDGFR, which binds all three PDGF isoforms and undergoes tyrosine autophosphorylation in response to each (8). By contrast, the previously identified PDGFR, designated the β receptor is preferentially tyrosine phosphorylated in response to the PDGF-BB isoform (dimer composed of two B chains; refs. 8–10). PDGF is known to induce a variety of functional responses, including DNA synthesis, chemotaxis, membrane ruffling, and inositol phospholipid breakdown in connective tissue cells (11, 12), which generally coordinately express both PDGFRs (8). Thus, to investigate functional responses mediated by each, we undertook efforts to independently express their cDNAs in a naive hematopoietic cell line devoid of either receptor (13). We show that each receptor can function in such cells and that each receptor independently mediates major known PDGF activities, including mitogenic signal transduction, chemotaxis, and stimulation of inositol phospholipid turnover. Their binding by different PDGF isoforms distinguishes the two receptor gene products functionally and establishes the recently identified α PDGFR as the preferred receptor for human PDGF.

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MATERIALS AND METHODS

Engineering Eukaryotic Expression Vectors. The open reading frames of human α and β PDGFR cDNAs (8) were cloned into the already described long terminal repeat no. 2 (LTR-2) expression vector (14). A *Bam*HI fragment including the entire open reading frame of the α PDGFR was cloned into the *Bam*HI site of the LTR-2 vector in the proper orientation. A *Sal* I–*Nde* I fragment of the β PDGFR was cloned into the *Xho* I site of the vector by use of *Sal* I linkers.

Transfection Assay. The interleukin 3 (IL-3)-dependent mouse hematopoietic cell line, 32D, has been described (15). DNA transfection of 32D cells was done by electroporation (13). Mass populations of stably transfected cells were selected by use of the selectable *Eco–gpt* marker in the LTR-2 expression vector. Cells expressing the *Eco–gpt* gene were selected by their ability to survive in the presence of a mycophenolic acid-containing medium (16).

Northern (RNA) Blot Analysis. Total cellular RNA was extracted as described (17), electrophoresed on a formaldehyde/agarose gel and transferred at 20 μg per lane to nitrocellulose filters. Filters were hybridized at 42°C in a buffer [40% (wt/vol) formamide/5× SSC (1× SSC is 0.15 M sodium chloride/0.015 M, sodium citrate), pH 7.0/1× Denhardt's solution (0.02% bovine serum albumin/0.02% Ficoll/0.02% polyvinylpyrrolidone)/5 mM NaH_2PO_4/5 mM Na_2HPO_4/0.1% SDS/salmon sperm DNA (100 μg/ml) containing ^{32}P-labeled cDNA probes prepared with a nick-translation kit (Amersham; ≈10^9 cpm/μg of DNA). After 16-hr hybridization, filters were washed twice for 20 min in 2× SSC at room temperature, then washed 30 min in 0.1× SSC/0.1% SDS at 50°C, and autoradiographed at −70°C.

Immunoblot Analysis. Cells were washed twice with phosphate-buffered saline/1 mM EGTA. The cell pellet was resuspended in 20 vol of hypotonic buffer (10 mM Hepes, pH 7.3/1 mM $MgCl_2$/1 mM phenylmethylsulfonyl fluoride/0.1% aprotinin) and lysed for 10 min at 4°C. The lysate was centrifuged at 1000 × *g* for 10 min, and the resulting supernatant was centrifuged for 90 min at 100,000 × *g*. The pellet (P100 fraction) was solubilized with staph-A buffer as described (8). The P100 lysate (150 μg/lane) was subjected to SDS/7% PAGE, electrophoretically transferred to a nitrocellulose filter, and probed with either anti-α PDGFR or

Abbreviations: PDGF, platelet-derived growth factor; PDGF-AB, -BB, and -AA, PDGF dimers composed of an A and a B chain, two B chains, and two A chains, respectively; PDGFR, PDGF receptor; IL-3, interleukin 3; $[Ca^{2+}]_i$, intracellular calcium concentration; LTR-2, long terminal repeat no. 2.

anti-β PDGFR peptide antibody (with or without the respective immunizing peptide) and ^{125}I-labeled protein A. The filters were subjected to autoradiography.

Ligand-Binding Assay. PDGF-BB was labeled by the Bolton–Hunter method (specific activity, 80,000 cpm/ng) (18). Human PDGF-AB is composed predominantly of the AB isoform (5). To exclude labeling of any contaminating PDGF-BB, ^{125}I labeling was performed by the chloramine-T method (19), which labels the tyrosine present only on the PDGF A chain (specific activity, 50,000 cpm/ng). ^{125}I-labeled PDGF-AB or -BB binding to cells done as described (8).

Mitogenic Assay. 32D transfectants (3×10^5 cells per ml) were washed twice with phosphate-buffered saline and then plated in 24-well plates in RPMI 1640 medium/15% fetal calf serum with or without the appropriate PDGF ligand. PDGF-BB was obtained from Amgen; PDGF-AB was obtained from R & D Systems (Minneapolis). PDGF-AA was immunoaffinity purified from PDGF-AA-producing NIH 3T3 cells (20) or a gift of A. Thomason, Amgen. Cells were incubated for 20 hr followed by a further 4-hr incubation with [^{3}H]methylthymidine (5 μCi/well; 1 Ci = 37 GBq). The cells were then harvested by an automatic cell harvester. Filter discs were placed in a liquid scintillation mixture (Biofluor, NEN) and counted in a β counter.

Chemotaxis Assay. Cell migration was assayed by means of a modified Boyden chamber technique using Nucleopore filters (5-μm pore size), as described elsewhere (21). Cells were washed twice, suspended in Dulbecco's modified Eagle's medium, and added to the upper chamber. PDGF isoforms were diluted in the same medium and added in the lower chamber. After 4-hr incubation at 37°C, cells that had migrated to the lower chamber were collected by centrifugation, washed twice with phosphate-buffered saline, and lysed in 0.08% SDS. Cell number was estimated by protein determination with the Bio-Rad protein assay kit.

Inositol Phosphate Determinations. Cells were prelabeled for 48 hr with [^{3}H]*myo*-inositol at 10 μCi/ml (NEN) in RPMI 1640 medium supplemented with 15% fetal calf serum and interleukin 3, following a described procedure (22, 23). Prelabeling was terminated by washing the cells several times and incubating them for 4 hr in serum-free, interleukin 3-free Dulbecco's modified Eagle's medium. Incubation with different PDGF isoforms was then performed in 35-mm dishes at 37°C with LiCl. The reaction was terminated by adding ice-cold methanol, and inositol phosphates were extracted and separated as described (24). Total inositol phosphates were eluted from Dowex AG 1X8 columns (Bio-Rad) with 1.2 M ammonium formate/0.1 M formic acid.

Measurement of Intracellular-Free Ca^{2+}. Intracellular Ca^{2+} levels of 32D transfectants were measured with the fluorescent Ca^{2+} indicator fura-2 (25). Cells were washed with RPMI 1640 medium and then loaded with fura-2/acetoxymethyl ester (1 μM) at 37°C. After 30-min incubation, the cells were washed several times and resuspended in Krebs–Ringer medium buffered with Hepes (125 mM NaCl/5 mM KCl/1.2 mM $MgSO_4$/1.2 mM KH_2PO_4/2 mM $CaCl_2$/6 mM glucose/25 mM Hepes, pH 7.4). Measurements were carried out on 2-ml cell suspensions (2×10^6 cells per ml), and Ca^{2+} concentration was calculated on the fluorescence ratio at 340 nm/380 nm as described (26).

RESULTS

Independent Expression of α and β PDGFR cDNAs in 32D Hematopoietic Cells. Recent studies have shown that introduction of an expression vector for the epidermal growth factor receptor in 32D mouse hematopoietic cells, a line normally dependent upon IL-3 for proliferation and survival, led to effective coupling with epidermal growth factor mitogenic signal-transduction pathways (13). Thus, we sought to utilize 32D cells to investigate biological and biochemical responses that might be specific to each PDGFR gene product. To do so, α and β PDGFR cDNAs were introduced into the LTR-2 expression vector that contains a linked *Eco-gpt* selectable marker (14). 32D cells were then transfected with either construct by electroporation and selected in medium supplemented with mycophenolic acid. After 2 weeks in the selective medium, viable cultures were obtained.

To study the expression of each receptor cDNA, total RNA from representative transfectants, designated 32D-αR and 32D-βR, as subjected to Northern blot analysis. As shown in Fig. 1, neither PDGFR transcript was detectable in the parental 32D cells, even under relaxed hybridization conditions, which allowed detection of the respective mouse PDGFR transcripts in NIH 3T3 fibroblasts. In contrast, 32-αR and 32D-βR transfectants expressed abundant transcripts specific to human α and β PDGFR genes, respectively (Fig. 1). When membrane lysates of the transfectants were subjected to immunoblot analysis, anti-α PDGFR peptide serum detected 190-kDa and 140-kDa protein species in 32D-αR but not in 32D-βR cells. Moreover, immunizing peptides were specifically competitive with these proteins. Conversely, 32D-βR cells contained 180- to 200-kDa species, which were specifically detected by anti-β PDGFR serum. None of these protein species was present in control 32D cells. The respective sizes of the two receptors have been reported to vary somewhat in different cell types (8–10), presumably due to differences in glycosylation.

When we compared ^{125}I-labeled PDGF-BB binding to 32D-αR or 32D-βR cell lines, the binding curves were similar (Fig. 2*A*). Scatchard analysis revealed ≈50,000 receptors per

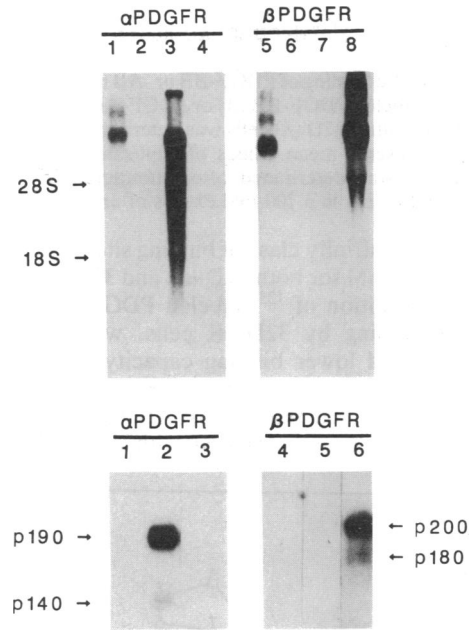

FIG. 1. Comparison of α and β PDGFR RNA and protein expression in 32D cell transfectants. (*Upper*) For determination of α and β PDGFR RNA expression, total RNA was extracted from NIH 3T3 (lanes 1 and 5), 32D control (lanes 2 and 6), 32D-αR (lanes 3 and 7), and 32D-βR (lanes 4 and 8) cells, electrophoresed in a 1% agarose gel, and subjected to RNA blot hybridization with ^{32}P-labeled α (lanes 1–4) or β (lanes 5–8) human PDGFR cDNA as probes. Filters were exposed to x-ray film with intensifying screens (lanes 2 and 4–7) for 24 hr or 4 hr (lanes 3 and 8). (*Lower*) For analysis of α and β PDGFR proteins, P100 fractions of 32D control (lanes 1 and 4), 32D-αR (lanes 2 and 5), and 32D-βR (lanes 3 and 6) cell lines were prepared and subjected to immunoblot analysis with peptide antiserum specific for each PDGF receptor (8). Arrows, α and β PDGFR protein species, confirmed by blocking with the immunizing peptide (data not shown).

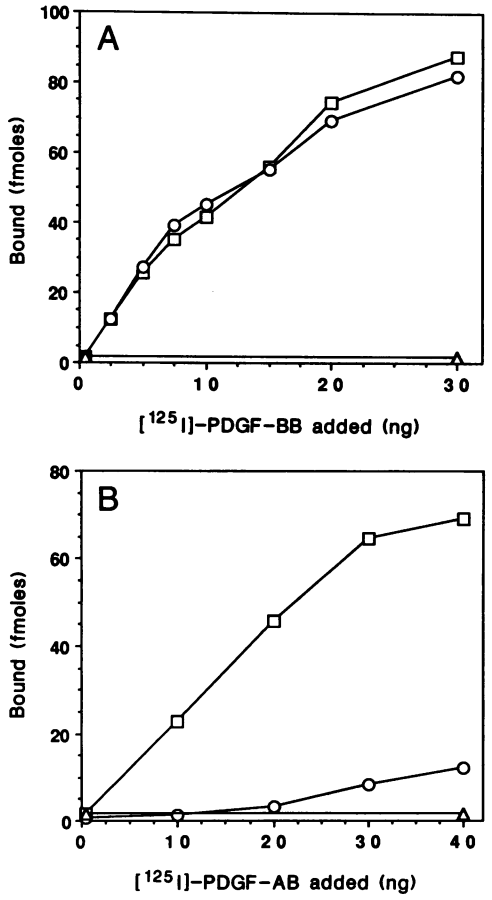

FIG. 2. Specific binding of PDGF-BB or -AB to 32D transfectants. Binding of ^{125}I-labeled PDGF-BB (*A*) or PDGF-AB (*B*) to 32D-αR (□), 32D-βR (○), or control 32D (△) cells was determined as described (8). Each point represents mean values of triplicate samples in which specific binding was determined after subtraction of ^{125}I-labeled PDGF isoform binding in a 100-fold excess of unlabeled ligand.

cell with a single affinity class of binding sites. The molecular masses were ≈1 nM for both 32D-αR and 32D-βR lines (data not shown). Addition of ^{125}I-labeled PDGF-AB resulted in preferential binding by 32D-αR cells, with 32D-βR cells showing ≈10-fold lower binding capacity (Fig. 2*B*). Standardized on the basis of their similar binding by PDGF-BB, these results indicated that PDGF-AB had a considerably lower affinity for β as compared with α PDGF receptors independently expressed by 32D cells. Under conditions where binding of ^{125}I-labeled PDGF-AA to 32D-αR cells was readily detectable, no specific binding to 32D-βR cells was observed (data not shown). The limited availability of PDGF-AA precluded Scatchard analysis. These results were consistent with binding properties of two PDGFR species expressed in fibroblasts (27–30).

Mitogenic Signal Transduction by α and β PDGFR Gene Products in 32D Cells. Proliferation of 32D cells is normally strictly dependent on IL-3 (15). Deprivation of IL-3 from the medium led to rapid loss of viability both of the transfectants and of the control 32D cells. To determine whether intracellular mitogenic signaling pathways for PDGF preexisted in 32D cells and whether ligand binding to α or β PDGFRs coupled with such pathways, we analyzed the abilities of different PDGF isoforms to induce DNA synthesis in IL-3-deprived 32D-αR or 32D-βR cell lines. As shown in Fig. 3*A*, PDGF-BB had no detectable mitogenic effect on control 32D cells. In contrast, this isoform coupled efficiently with mitogenic signal-transduction pathways in a similar dose-dependent manner in both 32D-αR and 32D-βR lines. Thus, expression in 32D cells of either α or β PDGFR gene products by 32D cells was both necessary and sufficient for induction of a potent mitogenic response to PDGF-BB.

Whereas 32D-αR cells were very responsive to PDGF-AB, 32D-βR cells showed significantly lower levels of [^{3}H]thymidine incorporation after PDGF-AB stimulation (Fig. 3*B*). These results were consistent with the lower affinity of PDGF-AB for the β PDGFR expressed in 32D-βR cells (Fig. 2*B*). Finally, PDGF-AA induced DNA synthesis by 32D-αR cells but did not cause any detectable mitogenic response in 32D-βR cells (Fig. 3*C*). That the level of thymidine incorporation reflected a sustained proliferative effect was confirmed by analysis of colony formation in semi-solid agar-containing medium. Both 32D-αR and 32D-βR cell lines readily formed colonies in PDGF-BB-supplemented medium, whereas only 32D-αR cells did so in medium supplemented with PDGF-AB (data not shown). Thus, proliferation of 32D-αR and -βR cell lines correlated with binding of the respective PDGF isoforms to α or β PDGFRs independently expressed by either cell line.

α and β PDGFRs Independently Induce Chemotaxis in Response to PDGF. Chemotaxis is another major functional response of fibroblasts and smooth muscle cells to PDGF (11, 21). Thus, we sought to investigate whether pathways for PDGF-mediated chemotaxis preexisted in 32D cells, as well as which of the PDGFR products performed this function. To do so, we devised a chemotaxis assay using a modified Boyden chamber technique (21). As shown in Fig. 4, PDGF-

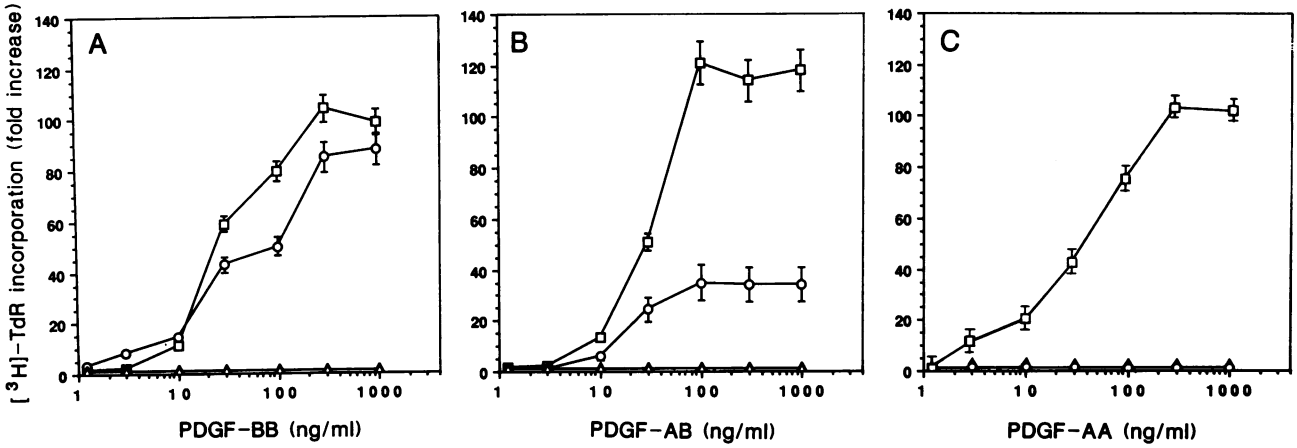

FIG. 3. Mitogenic response of 32D transfectants to different PDGF isoforms. DNA synthesis by 32D-αR (□), 32D-βR (○), and 32D (△) cells in response to PDGF-BB (*A*), PDGF-AB (*B*), or PDGF-AA (*C*) was measured by [^{3}H]thymidine incorporation in IL-3-free medium as described. Results are expressed as fold increase in [^{3}H]thymidine incorporation over that without ligand and are mean values of triplicate samples.

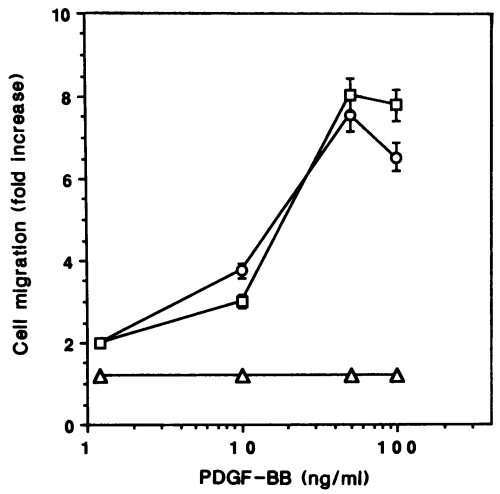

FIG. 4. Comparison of the chemotactic response of 32D-αR and 32D-βR cell lines to PDGF-BB. Cell migration of 32D-αR (□), 32D-βR (○), and 32D control cells (△) was assayed by means of a modified Boyden chamber technique using Nucleopore filters as described. Results are expressed as fold increase in cell migration compared with that seen using medium without PDGF-BB. Results represent the mean values of triplicate samples.

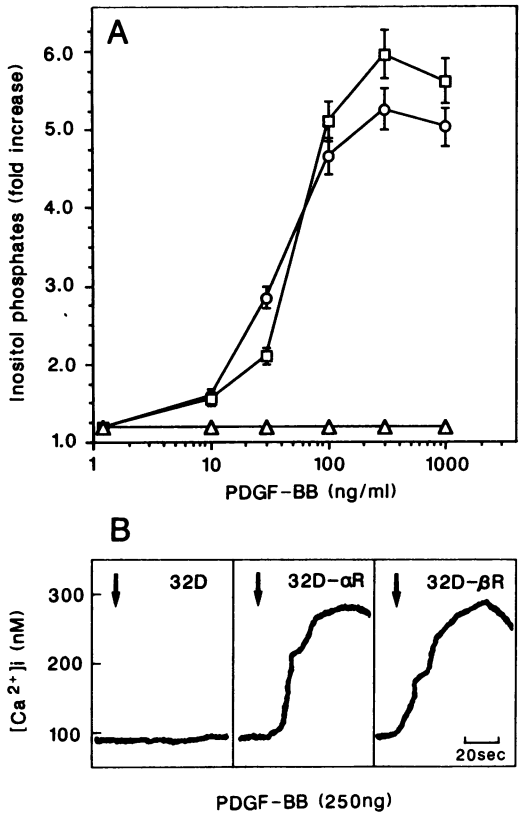

FIG. 5. Effect of PDGF-BB on inositol phosphate formation and cytosol-free Ca^{2+} mobilization in 32D-αR and 32D-βR transfectants. (*A*) 32D-αR (□), 32D-βR (○), and 32D control (△) cells were prelabeled with [^{3}H]*myo*-inositol for 48 hr and then exposed to varying concentrations of PDGF-BB. The reaction was stopped at 30 min, and total inositol phosphates were analyzed as described (24). Results are expressed as fold increase over unstimulated values and represent mean values of triplicate samples. (*B*) The fluorescent indicator fura-2 was used to determine cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) in 32D transfectants. Measurements were carried out on 2-ml samples in suspension (2×10^6 cells per ml) as described (25). Results show representative recordings of the fluorescence ratio 340 nm/380 nm. Arrows, times of PDGF-BB addition (250 ng/ml). $[Ca^{2+}]_i$ concentration was calculated on the 340 nm/380 nm ratio (26).

BB induced a readily measurable chemotactic response in both 32D-αR and 32D-βR cell lines. Checkerboard analysis revealed that 32D cell migration induced by PDGF-BB was due to directed cell migration (chemotaxis) and not to random migration (chemokinesis) (data not shown). Thus, both PDGFRs were able to independently couple with chemotactic as well as mitogenic signaling pathways inherently present in 32D cells. The relative effects of the three PDGF isoforms on chemotaxis in 32D-αR and -βR cells are shown in Table 1. It should be noted that human platelet PDGF is composed mainly of PDGF-AB but contains as much as 30% PDGF-BB as well (5). Thus, a component of the response seen in 32D-βR cells at very high levels may reflect contaminating PDGF-BB in the PDGF-AB preparation.

α and β PDGFRs Independently Couple with the Inositol Phospholipid Second-Messenger Pathway. There is substantial evidence that PDGF stimulation of fibroblasts induces rapid turnover of inositol phospholipids. This change results in the increase of second messengers, such as inositol 1,4,5-triphosphate, which mobilizes intracellular calcium ($[Ca^{2+}]_i$), and diacylglycerol, which activates protein kinase C (31, 32). To investigate the ability of α or β PDGFRs to couple with inositol phospholipid metabolic pathways, we challenged 32D-αR and 32D-βR lines with PDGF-BB. In each case, we could demonstrate accumulation of inositol phosphates as a function of time for up to 2 hr. When the responses of each cell line to stimulation with increasing amounts of PDGF-BB were analyzed at 1 hr, we observed a comparable PDGF-BB dose-dependent increase in inositol phosphates (Fig. 5*A*). Characterization by HPLC of inositol triphosphate isomers formed showed that inositol 1,4,5-triphosphate increased rapidly but transiently, whereas 1,3,4-triphosphate showed a slower increase and remained elevated. Inositol monophosphates continued to accumulate, so that they were the predominant species after 10 min (data not shown). No detectable differences were observed in the pattern inositol phosphate isomers formed in response to PDGF-BB triggering of either α or β PDGFRs expressed in 32D cells.

Table 1. Comparison of chemotaxis, inositol phospholipid turnover, and calcium influx in 32D-αR and -βR cells in response to different PDGF isoforms

32D transfectant	PDGF isoform	Chemotaxis, fold increase in cell migration		Inositol phospholipid turnover, -fold increase in Ins*P*		Calcium mobilization, $\Delta[Ca^{2+}]_i$
		10 ng/ml	300 ng/ml	250 ng/ml	1000 ng/ml	250 ng/ml
αR	AA	2.0	8.0	—	4.5	90 nM
βR		ND	ND	ND	ND	0
αR	AB	2.7	8.7	5.5	5.5	180 nM
βR		ND	5.1	ND	4.5	0
αR	BB	3.7	8.0	6.0	6.0	200 nM
βR		3.2	7.8	5.0	5.0	190 nM

Ins*P*, inositol phosphates; ND, no difference compared with levels in unstimulated cells; —, not tested.

$[Ca^{2+}]_i$ was also measured in 32D-αR or 32D-βR cells prelabeled with the fluorescent $[Ca^{2+}]_i$ indicator fura-2, followed by exposure to PDGF-BB at 250 ng/ml. Fig. 5*B* shows that control 32D cells were not detectably responsive. In contrast, 32D-αR and 32D-βR cell lines showed comparable, marked increases in $[Ca^{2+}]_i$ under the same conditions. All of these findings demonstrated the inherent capacities of α and β PDGFRs to independently couple effectively with the inositol phospholipid second-messenger pathway. Table 1 shows further that the magnitude of the inositol phospholipid response, as well as $[Ca^{2+}]_i$, correlated reasonably well with the binding properties of different PDGF isoforms to α and β receptors, respectively.

DISCUSSION

We have investigated specific functions mediated by the products of two independent PDGFR-encoding genes. By use of a strategy involving introduction of expression vectors for α and β PDGFR cDNAs into a naive hematopoietic cell line, we show here that each receptor could independently couple with mitogenic signal-transduction pathways inherently present in these cells. Moreover, both receptors could induce a readily detectable chemotactic response. Finally, activation of either receptor rapidly stimulated inositol phospholipid metabolism and mobilization of $[Ca^{2+}]_i$. All of these findings establish that the major biological and biochemical responses seen in cells normally triggered by PDGF can be reconstituted in these hematopoietic cells by expression of either α or β PDGFRs. The ability of these receptors to independently induce these responses argues that each receptor is inherently capable of doing so in the absence of interaction with the other receptor.

PDGF consists of AA, AB, and BB isoforms, which arise as dimeric products of two independent PDGF-encoding genes (1, 5, 33). The PDGF A chain is more ubiquitously expressed by normal cell types than the PDGF B chain (23) and is more actively secreted as well (20). Our present results indicate that a major level of regulation of the spectrum of PDGF functional responses resides in the relative affinities of the three PDGF isoforms for either receptor. PDGF-BB, which exhibited similar high affinity for binding α and β PDGFRs, induced each of the functional responses analyzed with comparable efficiency in 32D-αR and -βR cells. Similarly, human PDGF-AB and PDGF-AA, which bound the α receptor with much better affinity than the β receptor, induced functional responses preferentially in 32D-αR cells. Thus, the availability of specific PDGF isoforms and the relative expression of each receptor gene product appear to be major determinants of the PDGF response.

A recent report has indicated that an interkinase deletion mutant of the β PDGFR undergoes tyrosine phosphorylation and induces inositol phosphate formation but cannot elicit a mitogenic response (34). These findings have implied that the interkinase region may be critical for interaction with a substrate necessary for effective mitogenic signaling. More recent evidence has suggested that this substrate may be an unusual inositol phospholipid kinase (35). If so, the fact that α and β PDGFRs are highly divergent in their interkinase regions (8) raises the possibility that these regions may interact with distinct substrates. Thus, specific substrate availability could also differentially regulate the activities of the two PDGFs during development and/or in differentiated cell types that normally express both receptor genes.

As organisms have evolved, mechanisms of intercellular communication have evolved in complexity as well. The PDGF system represents an example of how gene duplications have given rise to more intricate regulation by growth factors and their receptors. It will be of interest to investigate the developmental expression of the related PDGF ligands and their receptors to gain further insights into the observed evolutionary divergence in this system. Concomitantly, availability of molecular and immunologic probes for each component of this complex growth factor-receptor system should aid in exploring the specific contributions of each component to those diseases in which aberrant PDGF expression is implicated.

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