Platelet-derived growth factor (PDGF)-AB-mediated phosphorylation of PDGF β receptors

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Platelet-derived growth factor (PDGF) stimulates the proliferation of Balb/c-3T3 fibroblasts through binding and subsequent activation of PDGF receptors. Activation of the PDGF receptors has been proposed to involve receptor dimerization. PDGF-AB has been shown to bind PDGF α and β receptor subunits to form PDGF $\alpha\beta$ and $\alpha\alpha$ receptor dimers. In this paper we demonstrate

that, following the down-regulation of PDGF α receptors, the binding of PDGF-AB to β receptors occurred at 37 °C but not at 4 °C. PDGF-AB stimulated the phosphorylation of PDGF β receptor monomers in cells depleted of PDGF α receptors by prior exposure to PDGF-AA.

INTRODUCTION

Platelet-derived growth factor (PDGF) is a potent mitogen for cells of mesenchymal origin, and stimulates the proliferation of density-arrested Balb/c-3T3 fibroblasts (Pledger et al., 1978; Williams et al., 1982; Sturani et al., 1989; Coats et al., 1991). PDGF was first purified from human platelets and shown to consist of a heterodimer composed of two disulphide-linked polypeptide chains, designated A and B (PDGF-AB) (Antoniades et al., 1979; Heldin et al., 1979; Raines and Ross, 1982). Subsequently, two additional homodimeric isoforms of PDGF were isolated, composed of either two A chains (PDGF-AA) or two B chains (PDGF-BB) (Johnsson et al., 1984; Heldin et al., 1986; Hammacher et al., 1988). Two receptors for PDGF, designated α and β , have been identified and shown to be ligand-activated tyrosine kinases (Yarden et al., 1986; Claesson-Welsh et al., 1988; Matsui et al., 1989a,b; Olashaw et al., 1991). The PDGF-B chain binds the PDGF β and α receptors with high affinity, whereas the PDGF-A chain only recognizes PDGF α receptors (Heldin et al., 1979; Gronwald et al., 1988; Claesson-Welsh et al., 1989).

It is proposed that PDGF α and β receptors dimerize in the presence of ligand to form three classes of activated receptors, designated $\alpha\alpha$, $\alpha\beta$ and $\beta\beta$ (Hammacher et al., 1989; Seifert et al., 1989; Li and Schlessinger, 1991; Ueno et al., 1991). All three PDGF isoforms would be capable of binding and dimerizing α receptors, while PDGF-BB would be the only isoform capable of binding with high affinity and inducing the dimerization of two monomeric β receptors. PDGF-AB could bind with high affinity and dimerize either two PDGF α receptor monomers or one α and one β receptor monomer to form both $\alpha\alpha$ and $\alpha\beta$ receptor dimers.

The receptor dimerization model predicts that PDGF-AB will not bind with high affinity to PDGF β receptors in the absence of α receptors (Hart et al., 1988; Matsui et al., 1989b), and data in support of this model have been presented (Seifert et al., 1989; Heidaran et al., 1991a,b; Kelly et al., 1991). In contrast, Hammacher et al. (1989) have demonstrated that PDGF-AB is capable of binding PDGF β receptors with low affinity, indicating that the B chain of PDGF-AB might interact monovalently with the

β receptor. In addition, Andersson et al. (1992) have demonstrated that a mutant B chain, which is incapable of forming PDGF-BB ligand dimers, binds monovalently and stimulates PDGF β receptor phosphorylation. In support of these findings, we have demonstrated that PDGF-AB stimulates a number of receptor-mediated events in density-arrested Balb/c-3T3 cells following the down-regulation of PDGF α receptors by PDGF-AA. These events include the down-regulation of PDGF β receptors, tyrosine phosphorylation of phospholipase C- γ (PLC- γ), induction of *c-fos* mRNA and stimulation of inositol phosphate formation (Drozdzoff and Pledger, 1991; Olashaw et al., 1991). PDGF-AB-mediated phosphorylation of PDGF β receptors and tyrosine phosphorylation of PLC- γ occurred in a temperature-dependent manner. In this paper we demonstrate that PDGF-AB binds and stimulates the tyrosine phosphorylation of monomeric PDGF β receptors at 37 °C but not 4 °C.

MATERIALS AND METHODS

Cell culture

Stock cultures of Balb/c-3T3 mouse fibroblasts (clone A31) were grown in Dulbecco–Vogt modified Eagle’s medium supplemented with 10 % calf serum, 4 mM L-glutamine, 50 units/ml penicillin and 50 μ g/ml streptomycin in humidified 5 % CO₂/95 % air. For all experimental procedures, cells were grown to confluency in serum-containing medium and used 2–3 days after cessation of growth.

PDGF binding assay

PDGF-AA and PDGF-AB were labelled with ¹²⁵I (New England Nuclear) using Iodo-beads (Markwell, 1982). For PDGF-AA binding studies, following the appropriate manipulations, cells in 35 mm-diam. dishes were placed on ice, rinsed once with ice-cold binding buffer [Hanks buffered saline solution buffered to pH 7.4 with 30 mM Hepes and supplemented with 0.25 % BSA (fraction V; Sigma)] and incubated for 3 h at 4 °C in 1 ml of binding buffer containing 3 ng/ml ¹²⁵I PDGF-AA. The cultures were then rinsed three times with binding buffer and solubilized with 1 ml of solubilization solution (1 % Triton-X 100, 0.1 % BSA). The

Abbreviations used: PDGF, platelet-derived growth factor; PLC- γ , phospholipase C- γ ; PPP, platelet-poor plasma; PMSF, phenylmethanesulphonyl fluoride.

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amount of cell-bound radioactivity was determined using a γ -radiation counter and was corrected for non-specific binding occurring in the presence of 200 ng/ml PDGF-AA. For PDGF-AB binding experiments, the same methods were used except that 2 ng/ml ^{125}I -PDGF-AB was used for binding assays and to correct for non-specific binding 200 ng/ml PDGF-BB was used. The ^{125}I -PDGF-AB binding assays were performed at either 37 °C for 10 min or 4 °C for 3 h, and specific binding was quantified as described above.

Immunoprecipitations

For pulse-chase experiments, cells in 35 mm-diam. dishes were incubated for 10–12 h in methionine-free medium containing 0.1 % platelet-poor plasma (PPP) and 100 $\mu\text{Ci/ml}$ [^{35}S]methionine (Amersham; sp. radioactivity > 800 Ci/mmol). Cells were then placed in medium containing the standard concentration of methionine (30 mg/l) and other ingredients as specified elsewhere in the text. Cells were rinsed with PBS (140 mM NaCl/2.6 mM KCl/8 mM Na_2HPO_4 /1.5 mM KH_2PO_4 , pH 7.4) and harvested by lysis in a Triton/glycerol solution [1 % Triton-X 100, 10 % glycerol, 1 mM phenylmethanesulphonyl fluoride (PMSF), 100 μM sodium vanadate, 1 mM NaF and 20 mM Hepes, pH 7.2; TG buffer]. The solubilized material was precleared with Pan-sorbin (Calbiochem) for 2 h at 4 °C, then incubated with rabbit PDGF α or β receptor antiserum at 4 °C for 4–8 h. Protein A-agarose (BRL) was added and the mixture was incubated for 2 h at 4 °C; the precipitated antibody-receptor complex was rinsed four times with radioimmune precipitation (RIPA) buffer (10 mM Tris, pH 8.0, 1.5 mM MgCl_2 , 150 mM NaCl, 1 mM EDTA, 1 % Triton X-100, 1 % deoxycholate, 0.1 % SDS and 1 mM PMSF) and heated to 95 °C in sample buffer (Laemmli, 1970). The beads were removed from the samples by centrifugation and the supernatant was analysed on 7.5 % SDS/polyacrylamide gels.

Immunoblots and cross-linking

Cells in 60 mm-diam. dishes were treated under various conditions as described later in the text, and either directly harvested or cross-linked prior to harvesting with 100 μM disuccinimidyl suberate (DSS) (Pierce) in PBS, pH 8.0. Cross-linking was performed for 15 min at either room temperature (for the binding assays performed at room temperature and 37 °C) or 4 °C (for the binding assays performed at 4 °C). Cells were harvested as described in the immunoprecipitation method section and cell lysates were heated to 95 °C in sample buffer and electrophoresed on SDS/PAGE gels. Proteins were transferred to nitrocellulose, blocked with blocking buffer (5 % non-fat dry milk, 50 mM Tris, pH 8.0, 0.2 % Nonidet P-40, 2 mM CaCl_2 and 80 mM NaCl) for 1 h at room temperature and incubated with antisera against phosphotyrosine or PDGF α or β receptors for 12–16 h at 4 °C. Immunoblots were rinsed and incubated for 1 h at room temperature with 4×10^5 c.p.m./ml of ^{125}I -labelled goat anti-mouse IgG (specific radioactivity 6.8 $\mu\text{Ci}/\mu\text{g}$; NEN) or ^{125}I -labelled goat anti-rabbit IgG (specific radioactivity 6.8 $\mu\text{Ci}/\mu\text{g}$; NEN) for detection of phosphotyrosine and PDGF receptor immunoblots respectively. Blots were rinsed extensively with blocking buffer with and without non-fat milk, dried and exposed to film for 12–24 h at –70 °C.

Antibodies

We expressed the C-terminal residues (amino acids 980–1089) from the mouse PDGF α receptor cDNA fused to glutathione S-

transferase in a pGEX expression vector. JM109 cells containing the pGEX1- α receptor plasmid were induced with 100 mM isopropyl thiogalactoside, solubilized and sonicated as previously described (Smith and Johnson, 1988). The fusion peptide was bound to glutathione-agarose and subsequently eluted from the agarose beads with 5 mM glutathione in 50 mM Tris/HCl, pH 8.0. New Zealand White rabbits were immunized with 0.2 mg of peptide in Freund's complete adjuvant and received three boosts at 1 month intervals with 0.1–0.2 mg of peptide in Freund's incomplete adjuvant; 10 days after the third boost, rabbits were bled and whole serum was obtained for use in immunoprecipitation and immunoblot analysis. This antibody does not recognize the PDGF β receptor. Anti-phosphotyrosine monoclonal antibody was purchased from UBI (Lake Placid, NY, U.S.A.).

Materials

Recombinant human PDGF-AA (> 95 % pure by SDS/PAGE) and PDGF-BB (> 95 % pure by SDS/PAGE) were obtained from Biosource Inc. (Westlake Village, CA, U.S.A.). Recombinant human PDGF-AB (PDGF-AB) (< 0.5 % of PDGF-AA and -BB) was generously provided by Arlen Thomason at Amgen Biologicals (Thousand Oaks CA, U.S.A.). PPP was made as previously described (Harrington et al., 1985). Protein A-agarose (BRL) was pretreated with a blocking solution of 1 % BSA (Pentex fraction V; Miles Pharmaceuticals) in PBS. PDGF β receptor polyclonal antiserum was generously provided by Dr Tom Daniel (Kumjian et al., 1989) (Vanderbilt University).

RESULTS

Down-regulation of PDGF α receptor protein

In order to maintain conditions which were as physiological as possible, we performed these studies in Balb/c-3T3 cells which possess both PDGF α and β receptors. To determine the ability of PDGF-AB to bind to PDGF β receptors, we first had to demonstrate that we were efficiently down-regulating all of the PDGF α receptors in Balb/c-3T3 fibroblasts. We used PDGF-AA to down-regulate the α receptors since it does not bind PDGF β receptors. To confirm that the PDGF α receptors were effectively down-regulated following PDGF-AA treatment, we performed PDGF α receptor immunoblots on cell lysates from fibroblasts treated with PDGF-AA in the presence or absence of cycloheximide. Figure 1(a) demonstrates that, following exposure of fibroblasts to PDGF-AA and cycloheximide for 1 h at 37 °C, all detectable α receptors were down-regulated. The presence of cycloheximide prevented the synthesis of PDGF α receptors, while in the absence of cycloheximide a small amount of α receptors was resynthesized in the presence of PDGF-AA.

To further verify efficient down-regulation of PDGF α receptors, we examined the ability of ^{125}I -PDGF-AA to bind fibroblasts pre-exposed to PDGF-AA. As shown in Figure 1(b), there was no detectable binding of ^{125}I -PDGF-AA at 4 °C in cells pretreated with PDGF-AA as compared with quiescent untreated cells. Under these conditions there was also a complete loss of ^{125}I -PDGF-AA binding at 37 °C (results not shown). When PDGF-AA-treated cells were washed free of PDGF and incubated at 37 °C in PPP-containing medium, ^{125}I -PDGF-AA binding fully recovered within 4–6 h (Figure 1b). This recovery required protein synthesis but not RNA synthesis (results not shown). In addition, we have performed PDGF α receptor immunoprecipitations to confirm that PDGF α receptors were completely down-regulated following exposure of cells to PDGF-AA for 1 h (see Figure 3; and results not shown). Immunoblot analysis indicated that PDGF-AA treat-

Figure 1 Down-regulation of PDGF α receptors

(a) Balb/c-3T3 fibroblasts were treated with medium containing PDGF-AA (50 ng/ml) in the presence or absence of cycloheximide (CHX; 1 $\mu\text{g}/\text{ml}$) for 30 min or 1 h at 37 $^{\circ}\text{C}$. Control cells were treated with medium alone in the presence or absence of cycloheximide for 1 h at 37 $^{\circ}\text{C}$. Cell lysates were analysed on SDS/7.5%-PAGE gels and PDGF α receptor immunoblots were performed as described in the Materials and methods section. The triangles represent increasing concentrations of total protein present in the cell lysates (25, 50 and 75 μg). (b) Balb/c-3T3 fibroblasts were treated with PDGF-AA (50 ng/ml) for 1 h at 37 $^{\circ}\text{C}$; cells were then rinsed and placed into medium containing 0.1% PPP and incubated at 37 $^{\circ}\text{C}$ for an additional 0.5, 2, 4 or 6 h. ^{125}I -PDGF-AA binding assays were performed at 4 $^{\circ}\text{C}$ as described in the Materials and methods section. ^{125}I -PDGF-AA binding was determined immediately following PDGF-AA treatment (time zero) and during the recovery period, and compared with the level of ^{125}I -PDGF-AA binding present on untreated fibroblasts (Q).

ment did not alter the amount of PDGF β receptor protein (see below), demonstrating that PDGF-AA-treated cells, although devoid of PDGF α receptors, maintained their β receptors. These data indicate that PDGF α receptors were efficiently down-regulated following a 1 h exposure of cells to PDGF-AA and that cycloheximide prevented their resynthesis.

Temperature-dependence of ^{125}I -PDGF-AB binding in the absence of PDGF α receptors

Previously published data demonstrate that PDGF-AB stimulates the tyrosine phosphorylation of PLC- γ at 37 $^{\circ}\text{C}$ but not at 4 $^{\circ}\text{C}$ in Balb/c-3T3 cells depleted of α receptors (Olashaw et al., 1991). To determine if this result could be explained by temperature-restricted binding of PDGF-AB to β receptors, we examined the ability of ^{125}I -PDGF-AB to bind fibroblasts at 37 $^{\circ}\text{C}$ and 4 $^{\circ}\text{C}$.

In these experiments, ^{125}I -PDGF-AB binding was determined both immediately following exposure of cells to PDGF-AA and at 2 h after the PDGF-AA had been removed. In all experiments which required PDGF-AB, we used a highly purified form of recombinant human PDGF-AB (Amgen) reported to contain no more than 0.2% of contaminating PDGF-AA and -BB (Heidaran et al., 1991b). To ensure that newly synthesized PDGF α receptors were not contributing to the pool of receptors available to bind

Figure 2 Temperature-dependent binding of ^{125}I -PDGF-AB in PDGF-AA-treated cells

Cells were incubated in medium containing 0.1% PPP, PDGF-AA (50 ng/ml) and cycloheximide (1 $\mu\text{g}/\text{ml}$) for 1 h at 37 $^{\circ}\text{C}$, rinsed and transferred to medium containing 0.1% PPP. ^{125}I -PDGF-AB binding was assayed at either 37 $^{\circ}\text{C}$ (■) or 4 $^{\circ}\text{C}$ (▨) immediately following the removal of the PDGF-AA present during the pretreatment period (time 0) or 2 h after exposure to recovery medium. Cells were treated for 1 h at 37 $^{\circ}\text{C}$ with PDGF-BB (50 ng/ml) at which time ^{125}I -PDGF-AB binding assays were performed at 37 $^{\circ}\text{C}$ (▤). The percentages represent specific binding as compared with those of control untreated fibroblasts (Q). For all ^{125}I -PDGF-AB binding assays the non-specific binding was 10–15% of total binding. All data points represent the average of three separate experiments. The S.D. was $\pm 5\%$ for all data points.

PDGF-AB at 37 $^{\circ}\text{C}$, ^{125}I -PDGF-AB binding was assayed in the presence of cycloheximide and excess PDGF-AA. PDGF-AA competed with ^{125}I -PDGF-AB for any residual α receptor binding, whereas cycloheximide blocked the synthesis of α receptors during and following their down-regulation.

Figure 2 demonstrates that following a 1 h exposure of cells to PDGF-AA and cycloheximide at 37 $^{\circ}\text{C}$, the binding of ^{125}I -PDGF-AB at 4 $^{\circ}\text{C}$ was decreased by 86% (zero time) as compared with that in quiescent control cells. In contrast, the binding of ^{125}I -PDGF-AB at 37 $^{\circ}\text{C}$ in PDGF-AA-pretreated cells was reduced by only 29% (zero time). Under these conditions ^{125}I -PDGF-AB was shown to bind β receptors, as its binding at 37 $^{\circ}\text{C}$ was markedly reduced after treatment with PDGF-BB, which down-regulates both PDGF α and β receptors. Following the removal of PDGF-AA and cycloheximide, the binding of ^{125}I -PDGF-AB, at both 37 $^{\circ}\text{C}$ and 4 $^{\circ}\text{C}$, started to return within 2 h and was fully recovered by 6 h (Figure 2; and results not shown). The recovery of ^{125}I -PDGF-AB binding corresponded with the return of PDGF α receptors following exposure of cells to PDGF-AA (Figure 1b). These data show that PDGF-AB is able to find cells devoid of α receptors in a temperature-dependent fashion.

PDGF-AB binding at 4 $^{\circ}\text{C}$

The ability of receptors to be internalized and degraded is temperature-dependent; therefore PDGF-AA is capable of binding, but not down-regulating, PDGF α receptors at 4 $^{\circ}\text{C}$. We took advantage of the lack of receptor internalization at 4 $^{\circ}\text{C}$ to determine if the decrease in ^{125}I -PDGF-AB binding observed at 4 $^{\circ}\text{C}$ was dependent upon the process of PDGF α receptor internalization.

^{125}I -PDGF-AB binding assays were performed at 4 $^{\circ}\text{C}$ in cells pretreated with PDGF-AA at either 4 $^{\circ}\text{C}$ or 37 $^{\circ}\text{C}$. As indicated in Figure 3, pretreatment with PDGF-AA at either temperature was sufficient to decrease the subsequent binding of ^{125}I -PDGF-AB at 4 $^{\circ}\text{C}$ as compared with untreated control cells. In both instances, ^{125}I -PDGF-AB binding was reduced 85–95%

Figure 3 Internalization of PDGF α receptors is not required for decreased binding of ^{125}I -PDGF-AB at 4 °C

Cells were incubated in medium containing 0.1 % PPP and PDGF-AA (50 ng/ml) for 1 h at either 37 °C or 4 °C. ^{125}I -PDGF-AB binding assays were then performed at 4 °C and compared with the binding present on untreated cells (Q). The binding data represent the averages of three separate experiments. The inset depicts PDGF α receptor immunoprecipitations from Balb/c-3T3 fibroblasts which were prelabelled with [^{35}S]methionine, rinsed and placed into medium containing unlabelled methionine and 0.1 % PPP in the absence (lane 1) or presence (lanes 2 and 3) of PDGF-AA and incubated for 1 h at either 37 °C (lane 2) or 4 °C (lane 3). Cell lysates were immunoprecipitated with PDGF α receptor antiserum and analysed on SDS/7.5%-PAGE gels. The position of the 200 kDa molecular mass marker protein is indicated.

compared with that in control cells. The inset of Figure 3 demonstrates that while the PDGF α receptors were down-regulated in cells treated with PDGF-AA at 37 °C (lane 2), their levels were not altered in cells exposed to PDGF-AA at 4 °C (lane 3). Therefore cells exposed to PDGF-AA at 4 °C contained α receptors that were occupied with PDGF-AA and not available to bind PDGF-AB. These data indicate that the inability of PDGF-AB to bind (at 4 °C) cells exposed to PDGF-AA was not dependent upon processes involved in PDGF α receptor internalization.

Dimerization of PDGF receptors

The receptor dimerization model predicts that PDGF-AB requires α receptors to induce receptor dimerization and activation. However, we have demonstrated that PDGF-AB is capable of binding at 37 °C to Balb/c-3T3 fibroblasts depleted of α receptors. These data lead to the conclusion that PDGF-AB is binding to the β receptor; therefore we wished to determine whether PDGF-AB was binding to and stimulating the phosphorylation of the monomeric or dimeric form of the PDGF β receptor.

Using PDGF α receptor immunoblot analysis and receptor cross-linking, we confirmed that all three isoforms of PDGF were capable of forming α receptor dimers at either 37 °C or 4 °C in untreated quiescent fibroblasts (results not shown). Using this assay system we were unable to determine if the PDGF α receptor dimers formed in the presence of PDGF-AB were comprised of $\alpha\alpha$ or $\alpha\beta$ receptor subunits; however, others have demonstrated that PDGF-AB mediates the formation of both $\alpha\alpha$ and $\alpha\beta$ receptor dimers in fibroblasts (Hart et al., 1987; Seifert et al., 1989).

To determine if PDGF-AB were capable of dimerizing β receptors, PDGF-AB cross-linking analysis was performed on control cells and cells pretreated with PDGF-AA. Figure 4 shows that PDGF-BB was able to dimerize β receptors in the

Figure 4 Dimerization of PDGF β receptors

Balb/c-3T3 cells were incubated in medium containing 0.1 % PPP in the absence or presence of PDGF-AA (50 ng/ml) and cycloheximide (1 $\mu\text{g}/\text{ml}$) for 1 h at 37 °C. PDGF-AB and -BB binding assays were performed for 3 h at 4 °C or for 20 min at room temperature (R) (approx. 22 °C). Cycloheximide was included in the binding buffer to prevent resynthesis of PDGF α receptors following their down-regulation. Cross-linking was performed as described in the Materials and methods section, and cell lysates were analysed on 4–9 % linear gradient SDS/PAGE gels. Proteins were transferred to nitrocellulose and immunoblotted with PDGF β receptor antiserum. The position of the 180 kDa molecular mass marker protein is indicated. The monomeric and dimeric forms of the PDGF β receptor are indicated with bars on the right.

absence or the presence of α receptors at both room temperature and 4 °C. PDGF-AB stimulated β receptor dimerization in untreated fibroblasts at room temperature and 4 °C, but did not dimerize β receptors at either temperature in cells depleted of PDGF α receptors by pretreatment with PDGF-AA (Figure 4). The PDGF binding assays were performed at room temperature to decrease the rate of ligand-dependent receptor internalization and degradation observed at 37 °C, thereby enabling a larger number of receptors to be cross-linked. Similar results were obtained when PDGF-mediated β receptor dimerization assays were performed at 37 °C (results not shown). The binding of ^{125}I -PDGF-AB at room temperature in PDGF-AA-pretreated fibroblasts was comparable with the binding observed at 37 °C (Figure 2; and results not shown). Our data clearly show that, although ^{125}I -PDGF-AB was capable of binding (at 37 °C or room temperature) cells depleted of α receptors by pre-exposure to PDGF-AA, PDGF-AB did not induce β receptor dimerization.

Phosphorylation of PDGF β receptor monomers

In order to determine if PDGF-AB stimulated the tyrosine phosphorylation of PDGF β receptor monomers, the following experiments were performed. Balb/c-3T3 fibroblasts were treated with medium containing 0.1 % PPP in the presence or absence of PDGF-AA (50 ng/ml) and cycloheximide (1 $\mu\text{g}/\text{ml}$) for 1 h at 37 °C. We have previously shown that following a 1 h exposure to PDGF-AA, the α receptors are down-regulated and cells no longer respond to PDGF-AA, in terms of α receptor phosphorylation (Figure 1; Drozdoff and Pledger, 1991).

First, to compare the PDGF-AB-mediated tyrosine phosphorylation of β receptors in control fibroblasts and fibroblasts depleted of α receptors, untreated cells and cells pretreated with PDGF-AA (as described in Figure 1) were exposed to PDGF-AB or -BB at 37 °C or 4 °C. PDGF β receptor immunoprecipitations were performed and tyrosine phosphorylated proteins were detected by anti-phosphotyrosine immunoblot analysis. As demonstrated in Figure 5(a), both PDGF-AB and -BB were capable of stimulating the tyrosine phosphorylation of PDGF β receptors at 37 °C and 4 °C in untreated control cells.

Figure 5 Phosphorylation of PDGF receptors

(a) Balb/c-3T3 cells fibroblasts were incubated in medium containing 0.1% PPP in the absence or presence of PDGF-AA (50 ng/ml) and cycloheximide (1 μ g/ml) for 1 h at 37 °C. Cells were rinsed and exposed to PDGF-AB (75 ng/ml) or -BB (75 ng/ml) for 1 h at 4 °C or 10 min at 37 °C. Results are compared with those in control cells treated with medium alone (C1) or cells treated with PDGF-AA and cycloheximide for 1 h at 37 °C (C2). Cell lysates were immunoprecipitated with PDGF β receptor antiserum, electrophoresed on SDS/7.5%-PAGE gels and anti-phosphotyrosine immunoblots were performed as described in the Materials and methods section. (b) Cells were incubated in medium containing 0.1% PPP in the absence or presence (Prt. AA) of PDGF-AA and cycloheximide for 1 h at 37 °C. Cells were rinsed and exposed to PDGF-AB (75 ng/ml) or -BB (75 ng/ml) for 10 min at 37 °C. Cross-linking was performed for 15 min at 4 °C as described in the Materials and methods section, cells were solubilized, electrophoresed on 4–9% linear gradient SDS/PAGE gels and immunoblotted with anti-phosphotyrosine monoclonal antibody. Results are compared with those in control untreated cells (Q). The position of the 180 kDa molecular mass marker protein is indicated for both parts of the figure.

However, in fibroblasts pretreated with PDGF-AA, PDGF-AB was unable to stimulate the tyrosine phosphorylation of PDGF β receptors at 4 °C. In contrast, PDGF-BB stimulated PDGF β receptor phosphorylation at both temperatures in cells depleted of α receptors. These results support our binding data of Figure 2 which demonstrated that the binding of PDGF-AB to PDGF β receptors was temperature-dependent.

In order to determine whether the tyrosine phosphorylated PDGF β receptors shown in Figure 5(a) represented monomers and dimers, fibroblasts were depleted of α receptors and PDGF cross-linking analysis was performed. Briefly, control cells and cells pretreated with PDGF-AA were incubated with PDGF-AB or -BB for 10 min at 37 °C, cells were rinsed with PBS and cross-linking was performed as described in the Materials and methods section. Whole-cell lysates were electrophoresed on 4–9% linear gradient SDS/PAGE gels and tyrosine phosphorylated proteins were detected by anti-phosphotyrosine immunoblot analysis.

Figure 5(b) demonstrates that PDGF-AB-induced tyrosine phosphorylation was detectable in both PDGF receptor monomers and dimers at 37 °C in untreated cells, but was only detectable in β receptor monomers in cells devoid of α receptors. As expected, PDGF-BB was able to stimulate the tyrosine phosphorylation of PDGF receptor monomers and dimers in control and PDGF-AA-pretreated cells. We did not detect tyrosine phosphorylated receptors in untreated fibroblasts. The

data in Figure 5 demonstrate that PDGF-AB-mediated tyrosine phosphorylation was only detectable in PDGF β receptor monomers in cells depleted of α receptors.

DISCUSSION

Ligand-mediated activation of PDGF receptors is thought to function via a mechanism involving PDGF receptor dimerization. Binding studies and cross-linking experiments suggest that PDGF-AB is capable of binding to and dimerizing α and β receptor monomers to form active $\alpha\beta$ and $\alpha\alpha$ dimers. Accordingly, PDGF-AB is thought to activate the β receptor only in the presence of α receptors. In contrast to this model, we have found that addition of PDGF-AB to fibroblasts depleted of PDGF α receptors induces a number of signal transduction processes in a temperature-restricted manner, including autophosphorylation of the PDGF β receptor and tyrosine phosphorylation of PLC- γ .

Because of these observations, we examined the binding of PDGF-AB at 37 °C and 4 °C to cells under conditions where we have verified that the PDGF α receptors were down-regulated. In this paper we demonstrate that, following the down-regulation of PDGF α receptors, 125 I-PDGF-AB was capable of binding to Balb/c-3T3 fibroblasts at 37 °C but had a dramatically decreased capacity to bind at 4 °C (Figure 2). A 1:4 ratio of PDGF α and β receptors is present on Balb/c-3T3 fibroblasts (Drozdzoff and Pledger, 1991); therefore the 29% decrease in 125 I-PDGF-AB binding at 37 °C to cells pretreated with PDGF-AA presumably represents the loss of α receptor specific binding. It appears that occupancy of PDGF α receptors by PDGF-AA at 4 °C is sufficient to block the subsequent binding of PDGF-AB at 4 °C (Figure 3). However, PDGF-AB is capable of binding fibroblasts at 37 °C following PDGF-AA treatment that depletes PDGF α receptors (PDGF-AA at 37 °C) or that brings about the occupancy of α receptors (PDGF-AA at 4 °C) (results not shown). Our data are supported by the recent findings of Seifert et al. (1993), demonstrating that PDGF-AB stimulated phosphorylation of the PDGF β receptors in fibroblasts derived from *Patch* (*Ph*) mutant mice which are genetically null for the PDGF α receptor. They demonstrate that the PDGF-AB-mediated receptor phosphorylation is temperature-dependent.

Our data agree with the receptor dimerization model proposed by Seifert et al. (1989) and Hammacher et al. (1989) in that PDGF-AB is not capable of dimerizing PDGF β receptors in the absence of α receptors. However, in contrast to the aforementioned reports, we have shown that PDGF-AB stimulated phosphorylation of the monomeric form of PDGF β receptors at 37 °C in cells pre-exposed to PDGF-AA (Figure 5). A possible explanation for the lack of detectable PDGF-AB-mediated β receptor dimerization in PDGF-AA treated cells is that, although the B chain of PDGF-AB can bind PDGF β receptor monomers, the β receptor subunits do not dimerize. Under these conditions the PDGF β receptor monomer subunits would be capable of transphosphorylating adjacent receptors in the absence of receptor dimerization at 37 °C but not at 4 °C. The elevated temperature would be required to allow the receptors to interact.

In contrast, Andersson et al. (1992) have demonstrated that monovalent PDGF B chains are capable of stimulating the phosphorylation and dimerization of PDGF β receptors exogenously expressed in porcine aortic endothelial cells. One difference between our results and the experiments described by Andersson et al. (1992) are the methods which were used to cross-link receptors. We cross-linked receptors in intact cells, while they performed their cross-linking assays using lysates from cells which had been pre-exposed to monovalent PDGF B

chain. Under the conditions used in their cross-linking assays, it is possible that the absence of intact membranes enabled the receptors to move freely in the solubilization buffer (cross-linking assays were performed at room temperature) and dimerize. We cannot explain the discrepancy between the ability of monovalent B chain to bind PDGF β receptors at 4 °C and the lack of PDGF-AB binding at 4 °C. In our experimental system, PDGF-BB bound (at 4 °C) cells which have been pretreated with PDGF-AA (results not shown). Therefore it is possible that the tertiary structure of the PDGF-AB molecule, but not PDGF-BB, inhibits the B chain part of the PDGF-AB ligand dimer from binding (at 4 °C) cells depleted of PDGF α receptors.

We have previously demonstrated by Scatchard analysis that the binding affinity (at 4 °C) of PDGF-AB for PDGF β receptors is 10-fold lower in the absence of α receptors (Drozdoﬀ and Pledger, 1991). The binding affinity of PDGF-BB for PDGF β receptors alone is not altered; therefore the ability of the PDGF B chain to bind PDGF β receptors at 4 °C may be dependent upon whether PDGF-B has formed a hetero- or homo-dimer with either the PDGF A or B chain. The inability of PDGF-AB to bind (at 4 °C) fibroblasts depleted of PDGF α receptors suggests that either the receptors or ligand are unable to make conformational changes required for binding and activation of PDGF β receptors alone. The high-affinity binding of PDGF-AB to PDGF α and β receptors at 4 °C in untreated cells would stimulate receptor dimerization.

The ability of PDGF-AB to stimulate the phosphorylation of PDGF β receptor monomers and activate second messenger systems supports the idea that PDGF-AB may be capable of mediating a mitogenic response in cells which only express PDGF β receptors. These findings have physiological significance in the sense that a number of cell types only express PDGF β receptors, while PDGF-AB is the predominant PDGF isoform present in human platelets. Therefore it is possible that PDGF-AB stimulates the growth of a wider range of cell types than previously considered.

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