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Isoform-specific induction of actin reorganization by platelet-derived growth factor suggests that the functionally active receptor is a dimer

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Human platelet-derived growth factor (PDGF) occurs as three isoforms which are made up of disulfide-bonded A and B chains. The isoforms bind with different affinities to two different but structurally related cell surface receptors. The A type receptor binds all three isoforms (PDGF-AA, PDGF-AB, PDGF-BB) with high affinity, whereas the B type receptor binds PDGF-BB with high affinity, PDGF-AB with lower affinity but does not appear to bind PDGF-AA. We have utilized the differential effects of the three isoforms on actin reorganization and membrane ruffling in human foreskin fibroblasts to probe the idea that ligand-induced receptor dimerization is associated with receptor activation. Actin reorganization was found to be induced only by PDGF-AB and PDGF-BB and is therefore likely to be mediated by the B type receptor. Simultaneous addition of PDGF-AA, or downregulation of the A type receptor blocked the effect of PDGF-AB but not that of PDGF-BB. This is compatible with a model by which PDGF-AB binds to and dimerizes one A and one B type receptor; PDGF-AB therefore requires A type receptors in order to be functionally active at physiological concentrations. In cells with downregulated A type receptors, high concentrations of PDGF-AB inhibited the effect of PDGF-BB on actin reorganization. We believe that this is due to a monovalent binding of PDGF-AB to the B type receptors which prevents PDGF-BB from dimerizing the receptors. We also found that PDGF-AB requires the presence of A type receptors in order to effectively downregulate B type receptors, and to induce autophosphorylation of the B type receptor. In conclusion, the present data support a model according to which PDGF is a bivalent ligand that activates its receptor by dimerization.

Key words: autophosphorylation/dimerization/down-regulation/foreskin fibroblast/isoforms/platelet-derived growth factor

Introduction

Human platelet-derived growth factor is an M_r 30 000 protein made up of dimers of A and B chains that are held together by disulfide bonds (for reviews see Ross *et al.*, 1986; Heldin and Westermark, 1989). The A and B chains are encoded by separate genes and are ~60% similar in amino acid sequence (Betsholtz *et al.*, 1986). All three possible isoforms of PDGF (PDGF-AA, PDGF-AB and PDGF-BB) have been identified and isolated from platelets or human tumor cell lines (Stroobant and Waterfield, 1984; Heldin *et al.*, 1986; Hammacher *et al.*, 1988). Cross-competition binding experiments using the three different isoforms of PDGF have revealed the existence of two separate PDGF receptor types which differ in ligand specificity (Hart *et al.*, 1988; Heldin *et al.*, 1988). The A type receptor binds all three isoforms with high affinity, whereas the B type receptor binds PDGF-BB with high affinity, PDGF-AB with low affinity, but does not appear to bind PDGF-AA. Cloning and nucleotide sequence analysis of the cDNAs of the two receptors have shown that they are structurally related (Yarden *et al.*, 1986; Claesson-Welsh *et al.*, 1989a; Matsui *et al.*, 1989). The extracellular part of each receptor can be divided into five Ig-like domains and each intracellular part contains a protein tyrosine kinase domain that is split by an intervening sequence of some 100 amino acid residues. The overall amino acid sequence identity is ~44% with the highest homology score in the kinase domains. The mature forms of the A and B type receptors are glycoproteins of M_r 170 000 and 180 000 respectively (Claesson-Welsh *et al.*, 1989b).

The mechanism by which binding of PDGF to the extracellular domain of the PDGF receptor activates the intracellular protein tyrosine kinase is not fully understood. Studies on purified PDGF B type receptor have indicated that the addition of PDGF-BB induces receptor dimerization parallel to the activation of the protein tyrosine kinase (Heldin *et al.*, 1989). Additional evidence for dimerization of PDGF receptors has recently been reported (Seifert *et al.*, 1989). Since PDGF is a dimer, it is conceivable that each molecule of PDGF binds two receptor molecules; given the ligand specificities of the two PDGF receptor types, and provided the receptors are freely diffusible in the plasma membrane, this model infers that PDGF-AB and PDGF-BB may dimerize both types of receptors, whereas PDGF-AA may only dimerize the A type receptor (cf. the hypothetical model in Figure 1A).

Previous studies have shown that PDGF purified from human platelets induces an acute reorganization of actin and plasma membrane ruffling in human foreskin fibroblasts (Mellström *et al.*, 1983, 1988; Nistér *et al.*, 1988). The present study shows that this effect is caused by PDGF-AB and PDGF-BB, but not by PDGF-AA; it is therefore likely to be mediated by the PDGF B type receptor. By manipulating the experimental system, we have obtained results in favor of the notion that the functionally active PDGF B type receptor is a dimer.

Results

Previous studies have shown that PDGF induces a rapid actin reorganization and membrane ruffling in human fibroblasts, which is conveniently demonstrated by staining filamentous

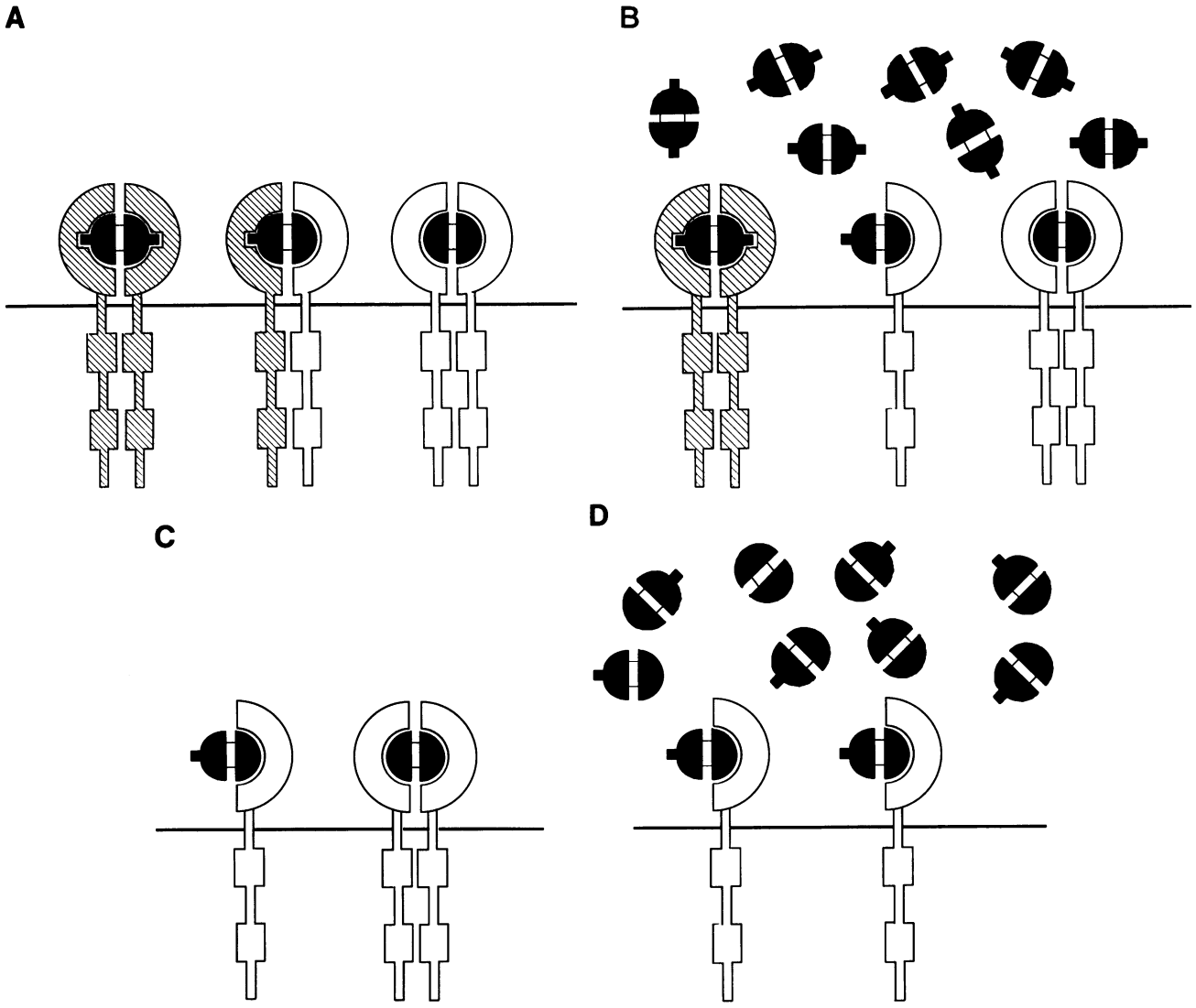

Fig. 1. Schematic representation of the hypothetical interaction of the three isoforms of PDGF with two receptor types [A type (hatched symbols) and B type (open symbols) receptor]. The model is based on the idea that PDGF is a bivalent molecule; one molecule of PDGF binds two receptor molecules. The specificities of monovalent interactions have been deduced by inference from previous binding experiments which have shown that the A type receptor binds to all three isoforms, whereas the B type receptor does not bind PDGF-AA. A chain, stalked half-circles; B chain, smooth half-circles. (**A**) Binding of PDGF to cells that have both types of receptors. For simplicity, the possibility that PDGF-BB may dimerize two A type receptors, and one A and one B type receptor, and that PDGF-AB may dimerize two A type receptors, has been omitted from the figure. (**B**) Binding of PDGF to cells in the presence of a saturating concentration of PDGF-AA. All A type receptors are blocked; PDGF-AB can therefore only bind monovalently; one molecule of PDGF-AB binds one B type receptor. Binding of PDGF-BB to the B type receptor is not affected since PDGF-AA has no affinity for this type of receptor. (**C**) Binding of PDGF-AB and PDGF-BB to cells in which the A type receptors have been down regulated. The situation is analogous to that presented in (B). (**D**) A saturating concentration of PDGF-AB blocks the binding of PDGF-BB in cells in which the A type receptors have been downregulated.

actin with rhodamine-conjugated phalloidin (Mellström *et al.*, 1988) (cf. Figure 2). The effect can be easily quantified by recording the number of cells displaying circular ruffle formations. In the present study we analyzed the abilities of the three isoforms of PDGF (PDGF-AA, PDGF-AB and PDGF-BB) to induce actin organization in serum-starved foreskin fibroblasts (line AG 1523) (Figure 3A). Both PDGF-AB and PDGF-BB were active in the assay, whereas the addition of PDGF-AA had no effect. The maximal level of stimulation obtained with PDGF-BB (60–90% responding cells) was consistently about twice that afforded by PDGF-AB (cf. Figure 3). This response pattern is consistent with the assumption that the motility effect of PDGF is mediated only via the B type receptor; this receptor does not recognize PDGF-AA.

As shown in Figure 1, our working hypothesis is that PDGF-AB may form a heterodimeric receptor complex, provided both types of receptors are present. In the absence of A type receptors, PDGF-AB only interacts via the B chain (the A chain is supposed to have only low affinity for the B type receptor) and can thus only form single ligand–receptor complexes. If the B type receptor is functionally active only in a dimeric configuration (cf. Heldin *et al.*, 1989), our model predicts: (i) that PDGF-AA should be a competitive inhibitor of PDGF-AB, but not of PDGF-BB, if both types of receptor are present (cf. Figure 1B); (ii) that downregulation of the A type receptor should abolish the effect elicited by PDGF-AB, but not by PDGF-BB (cf. Figure 1C); and (iii) that PDGF-AB, although functionally active on its own in naïve cells, should compete for the effect

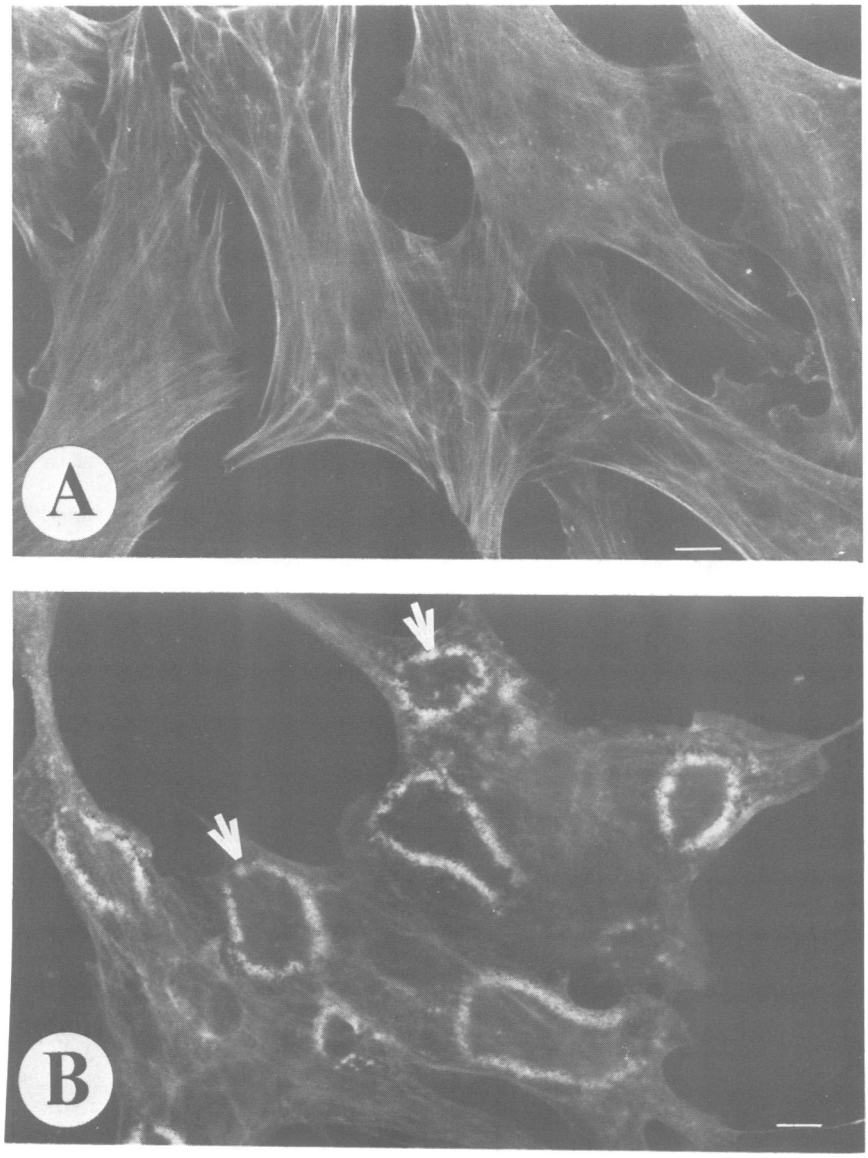

Fig. 2. Effect of PDGF on actin filament organization in human fibroblasts. Serum-starved foreskin fibroblasts were stained with rhodamine-conjugated phalloidin before (**panel A**) or after (**panel B**) exposure to 10 ng PDGF-BB/ml. Scale bar, 10 μm.

of PDGF-BB in cells in which the A type receptors have been downregulated (cf. Figure 1D).

These predictions have been tested in the following experiments.

As shown in Figure 3A, the simultaneous addition of 60 ng PDGF-AA/ml competed with PDGF-AB with regard to its stimulation of ruffle activity but had no effect on the response elicited by PDGF-BB. Moreover, pre-incubation at 37°C with 50 ng PDGF-AA/ml, a treatment known to effectively downregulate A type receptors, completely abolished the effect of PDGF-AB but did not influence the response to PDGF-BB (Figure 3B). As shown in Figure 4, the addition of increasing concentrations of PDGF-AB to cells with downregulated A type receptors partially blocked the effect of 10 ng PDGF-BB/ml. In conclusion, the results of these experiments agree with the view that the motility response is elicited by dimerized receptors, either in a heterodimeric or in a homodimeric configuration.

Considering that PDGF-AB binds to the B type receptor only with low affinity (K_d ~6 nM) (Severinsson *et al.*, 1989), it may seem surprising that this isoform is active in actin reorganization at relatively low concentrations (Figure 3A). In order to avoid interference with the A type receptor, binding experiments were performed on cells that lack endogenous expression of the A type receptor or had been pre-incubated with a saturating concentration of PDGF-AA to downregulate the A type receptor. We argue that the binding of PDGF-AB to the B type receptor might be of higher affinity in the presence of A type receptors that allow for a bivalent interaction, i.e. in a heterodimeric receptor complex (Figure 1A). This view is substantiated by the binding experiment depicted in Figure 5 which shows that PDGF-AB requires the presence of A type receptors in order to effectively downregulate B type receptors.

Studies using transfected PDGF B type receptor mutants have shown that the effect of PDGF on actin reorganization is linked to the protein tyrosine kinase activity of the receptor (B.Westermark, A.Siegbahn, C.-H.Heldin and L.Claesson-Welsh, unpublished); we therefore found it of interest to analyze receptor autophosphorylation in parallel to the

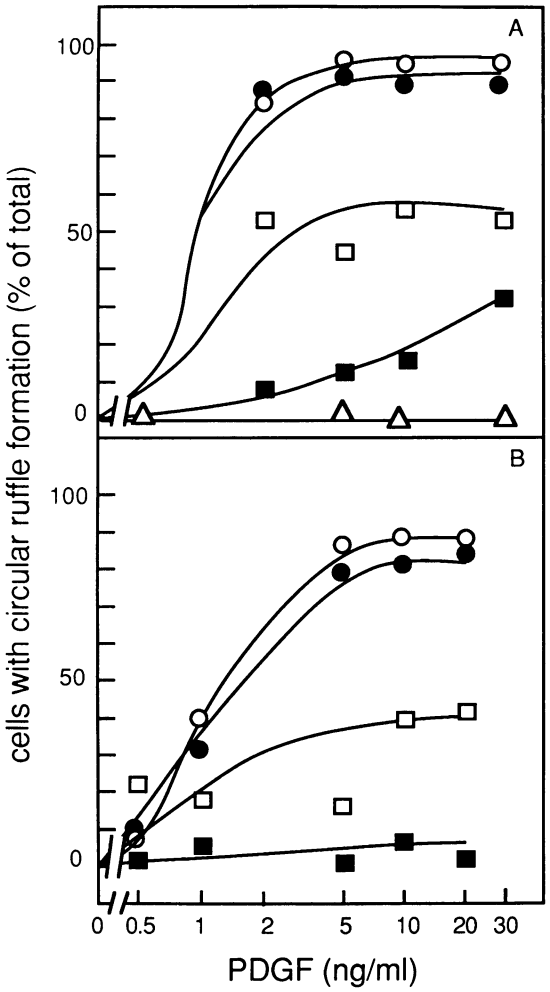

Fig. 3. Effect of the isoforms of PDGF on actin reorganization and membrane ruffling of human foreskin fibroblasts. The percentage of cells displaying circular ruffle formations was determined as described in Materials and methods. (**A**) Cells were incubated with various concentrations of PDGF-AA (triangles), PDGF-AB (squares) or PDGF-BB (circles), in the presence (closed symbols) or absence (open symbols) of 60 ng PDGF-AA/ml. (**B**) Cells were incubated with various concentrations of PDGF-AB (squares) or PDGF-BB (circles). Naïve cells (open symbols) or cells in which the A type receptors have been downregulated by 60 min incubation with 50 ng PDGF-AA/ml at 37°C (closed symbols) were used.

experiments described above. For this purpose, AG 1523 foreskin fibroblasts were metabolically labeled with [^{35}S] cysteine and [^{35}S]methionine and receptor autophosphorylation was estimated by immunoprecipitation with phosphotyrosine antiserum after incubation with a suboptimal concentration of PDGF-AB or PDGF-BB; both naïve cells and cells pre-incubated with PDGF-AA to downregulate the A type receptors were used in the experiment. As shown in Figure 6A, the phosphotyrosine antiserum brought down a 180 kd component from naïve cells stimulated with either PDGF-AB or PDGF-BB; the precipitation of this component was effectively blocked by a molar excess of phenyl phosphate. Downregulation of the A type receptor effectively inhibited PDGF-AB-induced autophosphorylation of the 180 kd B type receptor, whereas a PDGF-BB-induced autophosphorylation was much less affected.

In view of the finding that the A type receptor is a protein tyrosine kinase (Claesson-Welsh *et al.*, 1989a; Matsui *et al.*, 1989) that may become autophosphorylated by ligand binding

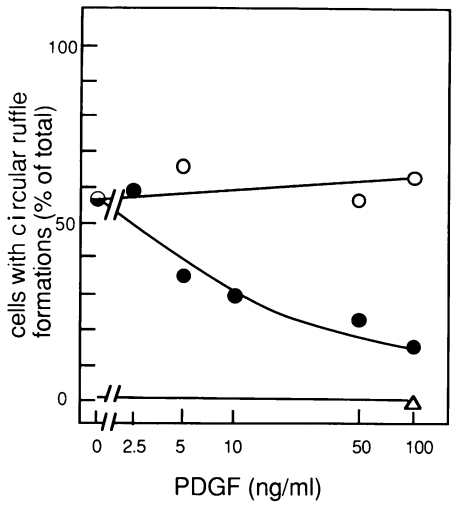

Fig. 4. Effect of downregulation of the A type receptor on PDGF-BB-induced membrane ruffling in foreskin fibroblasts. Cells were incubated with 10 ng of PDGF-BB and various concentrations of PDGF-AA (open circles), or PDGF-AB (closed circles) after downregulation of the A type receptors. Open triangle represents a control in which cells, with downregulated A type receptors, received only PDGF-AB.

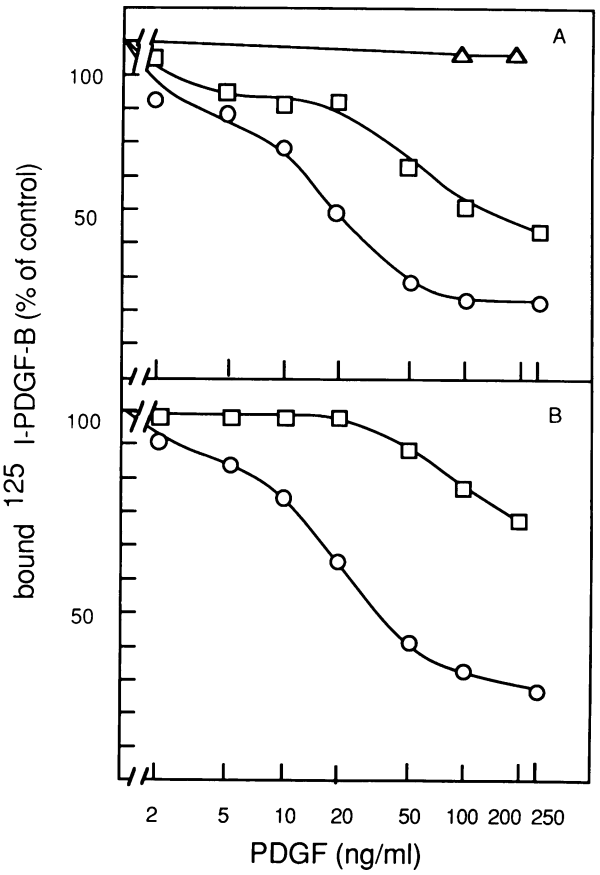

Fig. 5. Evidence that A type receptors are required for an effective differentiation of the B type receptor by PDGF-AB. [^{125}I]PDGF-BB binding was assessed after various regimens of PDGF receptor downregulation. In **panel A**, [^{125}I]PDGF-BB binding was studied after pre-incubation for 60 min at 37°C with various concentrations of PDGF-AA (triangles), PDGF-AB (squares) or PDGF-BB (circles). In **panel B**, cells were first incubated for 30 min wtih 50 ng PDGF-AA/ml. Before binding of [^{125}I]PDGF-BB, cells were further incubated for 60 min at 37°C with various concentrations of PDGF-AB (squares) or PDGF-BB (circles).

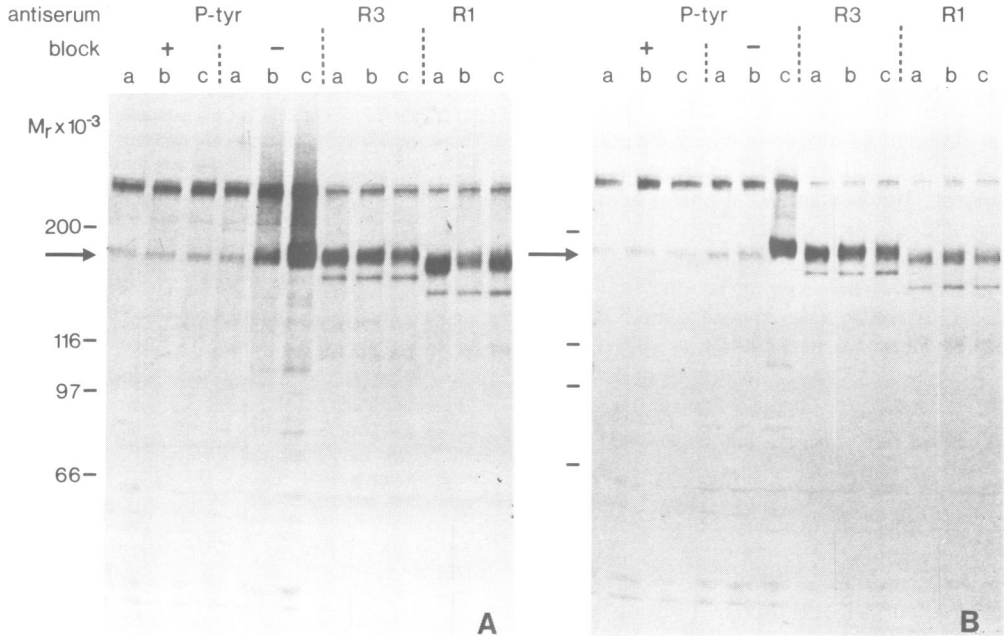

Fig. 6. Evidence that A type receptors are required for PDGF-AB-induced autophosphorylation of the B type receptor. Foreskin fibroblasts were metabolically labeled with [^{35}S]cysteine and [^{35}S]methionine and subjected to sequential immunoprecipitation using three rabbit antisera: P-tyr (antiphosphotyrosine), PDGFR-3 (specific for the B type receptor) and PDGFR-1 (recognizes both A and B type receptors). Naïve cells (**panel A**) or cells in which the A type receptors were downregulated (**panel B**) were used. Cells were stimulated for 6 min at 37°C with diluent (**lanes a**), 50 ng PDGF-AB/ml (**lanes b**) or 50 ng PDGF-BB/ml (**lanes c**). As a control of specificity, the phosphotyrosine antiserum was blocked with phenyl phosphate, as indicated in the figure.

(Hammacher *et al.*, 1989; Matsui *et al.*, 1989), it may seem surprising that the phosphotyrosine antiserum did not bring down any phosphorylated A type receptor from cells given PDGF-AA. It is possible that the amount of phosphorylated A type receptor was too low to render a signal under our assay conditions, either because of the low number of receptors, or because of a lower degree of phosphorylation of the A type receptor compared to the B type receptor.

A consecutive precipitation was then performed using PDGFR-3 antiserum, which specifically recognizes the B type receptor (Figure 6). As expected, the 180 kd B type receptor band brought down by this antiserum was somewhat fainter in cells incubated with PDGF-BB, in comparison with control cells; this difference reflects the fraction of autophosphorylated receptors brought down in the first precipitation. A third consecutive immunoprecipitation was performed using PDGFR-1 antiserum; this is directed against purified B type receptor but cross-reacts with the A type receptor. This antiserum brought down the expected 170 kd A type receptor species in naïve cells, whereas a considerably lower signal was recorded in cells pre-incubated with PDGF-AA; this result provides evidence for an effective downregulation of the A type receptor under these experimental conditions. In conclusion, the experiment indicates that PDGF-AB induces autophosphorylation of the B type receptor more efficiently when A type receptors are present.

Discussion

The functional characteristics of the two recently described PDGF receptor types have not yet been investigated in detail but available data indicate that their activities overlap to a great extent (for a review see Heldin and Westermark, 1989); this is not surprising considering their extensive structural similarity. However, previous and present data show that a motility response in human foreskin fibroblasts, including actin reorganization, the formation of circular membrane ruffles (Nistér *et al.*, 1988) and chemotaxis (Nistér *et al.*, 1988; A.Siegbahn, A.Hammacher, B.Westermark and C.-H.Heldin, unpublished) is only induced by B-chain-containing dimers, suggesting that these responses are mediated via the B type receptor only. Motivated by previous findings that PDGF-BB induces dimerization of purified B type receptors (Heldin *et al.*, 1989), we tried to utilize the differential effects on actin reorganization of the three isoforms to probe the idea that receptor dimerization also occurs *in vivo* and is associated with receptor activation. The leading principle in the design of the experiments was that PDGF is a bivalent ligand and that the A chain has affinity only for the A type receptor, whereas the B chain recognizes both types of receptors; the latter assumption is derived from binding experiments that show that PDGF-AA does not bind to the B type receptor (Hart *et al.*, 1988; Heldin *et al.*, 1988). Our model infers that PDGF-AB binds one A type and one B type receptor, or two A type receptors, and therefore should only be functionally active in the presence of A type receptors. This was shown to be the case both with regard to actin reorganization and receptor autophosphorylation. A crucial experiment was one that showed that PDGF-AB could either act as an agonist or as an antagonist, depending on the presence of A type receptors (Figure 4). The most likely explanation of this finding is schematically depicted in Figure 1D; in the absence of A type receptors, PDGF-AB binds monovalently to the B type receptor, thereby acting as a competitive inhibitor of PDGF-BB. In conclusion, our

data support the receptor dimerization model and are in accordance with the view that receptor dimerization is functionally linked to receptor activation.

As an alternative to the dimerization model presented above, one might envisage a situation in which the activated B type receptor is influenced by a negative signal mediated by the A type receptor. The fact that PDGF-AB is less potent than PDGF-BB in inducing the formation of circular ruffles in naïve cells (Figure 3) may be interpreted in this way. However, this model does not account for the inhibitory effect of PDGF-AB in cells with downregulated A type receptors (Figure 4); nor does it explain why PDGF-AB, but not PDGF-BB, requires A type receptors in order to effectively downregulate B type receptors (Figure 5), or to induce autophosphorylation of the B type receptor (Figure 6). Although a negative signal cannot be entirely ruled out, the lower efficiency of PDGF-AB compared to PDGF-BB with regard to actin reorganization can probably be ascribed to the fact that the cells express 3–4 times less A type than B type receptors (Östman *et al.*, 1989).

Growth factor receptors of the protein tyrosine kinase family have the same structural organization with a single stretch of amino acid residues that joins the ligand-binding extracellular domain to the intracellular catalytic domain (Yarden and Ullrich, 1988). An intramolecular mechanism of response activation thus requires that the conformational change of the extracellular portion of the receptor, brought about by ligand binding, be transmitted to the intracellular part through the transmembrane region. As this model infers an energetically unfavorable situation, Schlessinger (1988) has suggested that epidermal growth factor (EGF) stabilizes its receptor in a dimeric configuration, making a direct interaction between the intracellular domains possible. Direct evidence for this model has been presented (Cochet *et al.*, 1988). Unlike EGF, PDGF is a dimeric molecule and may thus induce receptor dimerization by a direct mechanism, not necessarily involving a conformational change of the extracellular domain. This model implies that monovalent PDGF should act as a PDGF antagonist. This is a potentially important aspect of our findings. It is generally believed that PDGF is involved in a variety of neoplastic and non-neoplastic disorders (for reviews see Ross *et al.*, 1986; Heldin *et al.*, 1987a), and the generation of PDGF antagonists may therefore be of clinical relevance.

Materials and methods

Cell culture

Human foreskin fibroblasts (line AG 1523) were purchased from the Coriell Institute, Camden, NJ. The cells were routinely grown in Eagle's minimum essential medium, supplemented with 10% fetal calf serum (Gibco) and antibiotics (100 IU of penicillin and 50 μg streptomycin/ml). Cultures were incubated at 37°C in a humidified atmosphere containing 5% CO_2. Only low passage (<20) cultures were used.

Purification and radiolabeling of PDGF

PDGF-AB was purified from human platelet lysates as described (Heldin *et al.*, 1987b; Hammacher *et al.*, 1988). PDGF-BB was either isolated from human platelets (Hammacher *et al.*, 1988) or purified from the conditioned medium of a yeast expression system (Östman *et al.*, 1989); recombinant PDGF-AA was also isolated from a yeast expression system. We could not detect any differences in potency or biological activity between platelet PDGF-BB and recombinant PDGF-BB. PDGF-BB was ^{125}I-labeled to a specific activity of 66 000 c.p.m./ng as described (Bolton and Hunter, 1973).

Fluorescence microscopy of PDGF-induced actin reorganization

Approximately 10^4 cells were seeded on glass coverslips contained in 35 mm Petri dishes. Cultures were incubated for 4 days in routine medium. Medium was then changed to serum-free MCDB 104, 0.5 mM Ca^{2+}. The assay for actin reorganization was performed 2–4 days later, as described (Mellström *et al.*, 1988). Briefly, samples to be tested were added and the cells then incubated on ice at 4°C for 60 min followed by incubation for 15 min at 37°C. The cells were fixed in freshly made 3% paraformaldehyde for 20 min at room temperature followed by 5 min in −20°C acetone. Actin was stained by a 20 min incubation with rhodamine-conjugated phalloidin (50 μg/ml) at room temperature. Coverslips were mounted in a 1:1 mixture of phosphate-buffered saline (PBS) and glycerol and viewed in a Leitz microscope equipped for epifluorescence. The number of cells displaying circular arrangements of actin was determined on coded specimens, by counting at least 100 cells per duplicate coverslip.

Assay for [^{125}I]PDGF-BB binding

For binding experiments, cells were seeded in 12 well cluster dishes and grown to confluence. The cells were then washed once with binding buffer (PBS containing 1 mg bovine serum albumin/ml) and further incubated for 1.5 h at 0°C in 0.5 ml binding buffer containing 1–2 ng of radiolabeled PDGF-BB. After three washes with ice-cold binding buffer, cell-associated radioactivity was solubilized in 0.5 ml of 1% Triton X-100 and determined in a gamma counter.

Immunoprecipitation of PDGF receptors from metabolically labeled cells

Cells forming a confluent monolayer in 100 mm Petri dishes (Falcon) were labeled in cysteine- and methionine-free MCDB medium, supplemented with [^{35}S]cysteine and [^{35}S]methionine (Amersham; sp. act. >800 Ci/mmol and 600 Ci/mmol respectively) for a total of 3.5 h. The labeled amino acids were added at concentrations of 50 μCi/ml each. After 2.5 h of labeling, diluent or 50 ng PDGF-AA/ml was added and the cells were incubated for another hour at 37°C. The cells were then stimulated with diluent or 50 ng/ml of either PDGF-AB or PDGF-BB for 6 min. For immunoprecipitations, cells were lysed in 0.15 M NaCl, 20 mM Tris, pH 7.5, 1% Triton X-100 (Merck), 0.1% SDS (BDH), 1% deoxycholate (Merck), 10 mM EDTA, 50 μM sodium orthovanadate (Sigma), 1 mM phenylmethylsulfonyl fluoride (Sigma), 1% Trasylol (Bayer, Leverkusen). The lysates were centrifuged at 10 000 *g* for 30 min at 4°C in an Eppendorf table-top centrifuge and the supernatants were pre-cleared by incubation with Protein A–Sepharose CL-4B (Pharmacia LKB Biotechnology Inc.) for 30 min at 4°C before addition of antiserum. The cleared lysates were divided into two equal parts and immunoprecipitated with phosphotyrosine antiserum with or without 40 mM phenylphosphate block. After treatment with Protein A–Sepharose, PDGFR-3 and PDGFR-1 were added sequentially to the supernatants from the material that had been reacted with unblocked phosphotyrosine antiserum. Typically, 10 μl of immune serum was added to each sample and incubated for 2–12 h at 4°C, followed by 50 μl (bead volume) Protein A–Sepharose for 30 min. The beads were washed 4 times in 0.15 M NaCl, 20 mM Tris, pH 7.5, 0,1% Triton X-100, 0.1% Tween 80 (Merck), 5 mg bovine serum albumin (Boehringer Mannheim)/ml and once in 20 mM Hepes, pH 7.4. Immune complexes were eluted by heating the beads for 5 min at 96°C in sample buffer containing 0.4% SDS, 0.2 M Tris, pH 8.8, 0.5 mM sucrose, 0.01% bromophenol blue (Merck), 20 mM dithiothreitol.

Electrophoresis

Reduced samples were electrophoresed in 5–12% gradient SDS–polyacrylamide slab gels (SDS-gels) (Blobel and Dobberstein, 1975). Chemicals were from Bio-Rad. Gels were prepared for fluorography by soaking in Amplify (Amersham), dried and exposed to Hyperfilm MP (Amersham).

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