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Different effects of homo- and heterodimers of platelet-derived growth factor A and B chains on human and mouse fibroblasts

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Binding sites for platelet-derived growth factor (PDGF) differ in their selectivity for the AA, AB and BB forms of PDGF. Human fibroblasts bind BB well and AA poorly, whereas Swiss 3T3 cells bind more similar quantities of each ligand. We found that AA PDGF was weakly mitogenic for human fibroblasts, but strongly mitogenic for 3T3 cells. Tyrosine phosphorylation of human fibroblast receptors was stimulated most by BB and least by AA, whereas the phosphorylation of 3T3 cell receptors was stimulated more uniformly by the three dimers. The receptor polypeptides that were phosphorylated were very similar. We suggest that phosphorylation of the receptor is proportional to the number of binding sites available for each ligand. Tyrosine phosphorylation of a number of other cell proteins was also proportional to receptor phosphorylation. In contrast, protein kinase C (PKC)-dependent serine and tyrosine phosphorylations were stimulated maximally by low level occupancy of PDGF binding sites, and phosphorylation of p36 required high occupancy. These data raise the possibility that differences in biological potency of AA, AB and BB forms of PDGF may be due simply to differences in the numbers of binding sites, rather than to different biochemical functions of their receptors.

Key words: PDGF receptor/protein-tyrosine kinase/phosphotyrosine/protein kinase C/calpactin

Introduction

Platelet-derived growth factor (PDGF) is composed of two disulfide-linked chains (A and B) with ~60% sequence identity (Betsholtz *et al.*, 1986). All three possible dimers of A and B chains exist naturally. Human platelet PDGF consists primarily of AB heterodimers, but also contains significant amounts of both homodimers (D.F.Bowen-Pope *et al.*, submitted). Platelets from many other mammals contain mostly the BB homodimer (Stroobant and Waterfield, 1984; D.F.Bowen-Pope *et al.*, submitted). Also, a protein closely related in sequence to the BB homodimer is synthesized by v-*sis* transformed cells (Devare *et al.*, 1983; Doolittle *et al.*, 1983; Waterfield *et al.*, 1983). The AA homodimer is secreted by certain human glioma (Nister *et al.*, 1988) and osteosarcoma cells (Heldin *et al.*, 1986). While human umbilical vein endothelial cells and osteosarcoma cells express both A and B chain transcripts, bovine endothelial cells contain primarily the B chain mRNA (Collins *et al.*, 1987b), and human diploid neonatal fibroblasts, primary skeletal myoblasts and adult arterial smooth muscle cells express the A chain but not the B chain (Sejersen *et al.*, 1986; Paulsson *et al.*, 1987; Sjölund *et al.*, 1988). These observations have led to speculation that the biological effects of the two chains and their three dimeric forms may differ.

High-affinity binding sites for platelet PDGF are found on cells of mesenchymal origin including fibroblasts, smooth muscle cells and glial cells. A PDGF receptor from human, porcine and murine sources has been purified based on the ability of human platelet PDGF to stimulate its intrinsic protein-tyrosine kinase activity (Daniel *et al.*, 1985; Bishayee *et al.*, 1986; Ronnstrand *et al.*, 1987). cDNA clones have been isolated for the human (Gronwald *et al.*, 1988; Escobedo *et al.*, 1988; Claesson-Welsh *et al.*, 1988) and mouse receptors (Yarden *et al.*, 1986). The cDNA sequences of the human and murine receptors are very similar, and typical of transmembrane receptor protein-tyrosine kinases (Yarden *et al.*, 1986; Gronwald *et al.*, 1988).

Different PDGF dimers bind unequally to human fibroblasts, suggesting that there are different categories of binding sites that differ in their relative binding affinities (Hart *et al.*, 1988; Heldin *et al.*, 1988; Nister *et al.*, 1988). One explanation of these data is that PDGF receptors are homo- or heterodimers of α and β subunits; α subunits bind either PDGF chain, and β subunits bind only B chains (Seifert *et al.*, submitted). Monoclonal antibody PR7212 binds to receptor β subunits (Hart *et al.*, 1988; Seifert *et al.*, submitted). Heldin *et al.* (1988) reported two types of receptors on human fibroblasts; one (type A) that binds AB, AA and BB PDGF with decreasing affinity, and one (type B) that binds BB well, AB poorly and AA not at all. These data are consistent with the receptor subunit model if type A receptors are $\alpha\alpha$ dimers and type B receptors are $\alpha\beta$ or $\beta\beta$ dimers. Monoclonal antibody PDGFR-B2 binds to type B receptors (Heldin *et al.*, 1988). These studies suggest that there is more than one type of PDGF binding site.

PDGF stimulates protein-tyrosine kinase activity in cell membranes and in the purified human receptor (Ek *et al.*, 1982; Nishimura *et al.*, 1982; Pike *et al.*, 1983). In intact cells, PDGF initiates tyrosine phosphorylation of the receptor and of several other cell proteins including p36 (calpactin I or lipocortin II) and a putative phosphatidylinositol kinase (Cooper *et al.*, 1982; Ek and Heldin, 1984; Frackelton *et al.*, 1984; Kaplan *et al.*, 1987). Another early event in PDGF-treated cells is increased phosphatidylinositol metabolism leading to the activation of the Ca^{2+}/phospholipid-dependent serine/threonine-specific protein kinase C (PKC) (Habenicht *et al.*, 1981; Nishizuka, 1984). This leads to the phosphorylation of many cellular proteins on serine

and threonine, and via some unresolved mechanism activates the serine and tyrosine phosphorylation of proteins known as p42 and p45 (Kazlauskas and Cooper, 1988; Vila and Weber, 1988). These early events are thought to contribute towards later changes including alterations in cell morphology, gene expression and cell proliferation (Heldin *et al.*, 1985; Ross *et al.*, 1986).

In this report we have compared the ability of the various PDGF dimers to induce DNA synthesis and intracellular phosphorylation events in cell types that bind AA, AB and BB differentially. We have found that, with the possible exception of p36 phosphorylation in Swiss 3T3 cells, all differences between the biological effects of the three PDGF dimers can be explained on the basis of receptor number. In addition, activation of PKC and subsequent PKC-dependent events require the phosphorylation of $< 10\%$ of the total fraction of PDGF receptors, irrespective of the PDGF dimer tested. Finally, while the PDGF binding sites are able to distinguish between the various dimers, the receptor polypeptides phosphorylated appear to be very similar in all cases.

Table I. Stimulation of [^{3}H]thymidine incorporation by different forms of PDGF

Cell type	PDGF		
	AA	AB	BB
AG1523	32 ± 05	112 ± 06	100
SK-5	29 ± 14	97 ± 07	100
3T3	110 ± 01	108 ± 01	100

Confluent, quiescent cells in 24-well dishes were incubated with a maximal dose of dimer (AA 4 ng/ml; AB 4 ng/ml; BB 16 ng/ml) at 37°C for 18 h, then 0.5 μCi/ml [^{3}H]thymidine was added and incubation continued for an additional 4 h at 37°C. Cell monolayers were first rinsed with ice-cold phosphate-buffered saline, then with 5% trichloroacetic acid, and the cell associated radioactivity was collected in 0.25 M NaOH and quantitated in a scintillation counter. The responses to AA and AB are expressed relative to BB as 100% ± SEM.

Results

In the following experiments, the effects of purified or crude recombinant human AA and BB homodimers were compared with the effects of immunoaffinity purified AB heterodimer, using three fibroblast cell lines. Since it was possible that a fraction of each PDGF preparation was biologically inactive, a saturating concentration of each was used, i.e. a 3-fold higher concentration of ligand did not alter the magnitude of any of the responses studied.

Mitogenicity of PDGF dimers

Mitogenic effects of AA, BB and AB dimers were compared using [^{3}H]thymidine pulse labeling. At the maximal dose of ligand [determined from a dose–response curve for each cell type with each dimer (Seifert *et al.*, submitted)], AA was a poor mitogen for both AG1532 and SK-5 fibroblasts relative to AB or BB, while in Swiss 3T3 cells all three dimers were similarly active (Table I). The mitogenic potency of AA PDGF for 3T3 cells suggests that this molecule is not inherently defective.

Since the mitogenic effect of human platelet PDGF is proportional to the quantity bound (Bowen-Pope and Ross, 1982; Frackelton *et al.*, 1984), differential binding of AA, AB and BB dimers could explain the unequal stimulation of DNA synthesis in human cells. Estimation of the receptor number by comparison of the quantity of radiolabeled ligand bound at saturation suggested that AG1523 and SK-5 fibroblasts have a much greater ratio of BB to AA binding sites, whereas in 3T3 cells there are just slightly fewer AA versus BB binding sites (Seifert *et al.*, submitted). In view of the inherent uncertainty in determining the fraction of radiolabeled ligand which is biologically active, the relative numbers of binding sites were also determined by a procedure independent of the specific activity of labeled ligand. The ratio of sites able to bind AA relative to those able to bind BB can be estimated by determining the fraction of total BB binding which is inhibited by the preincubation of cells

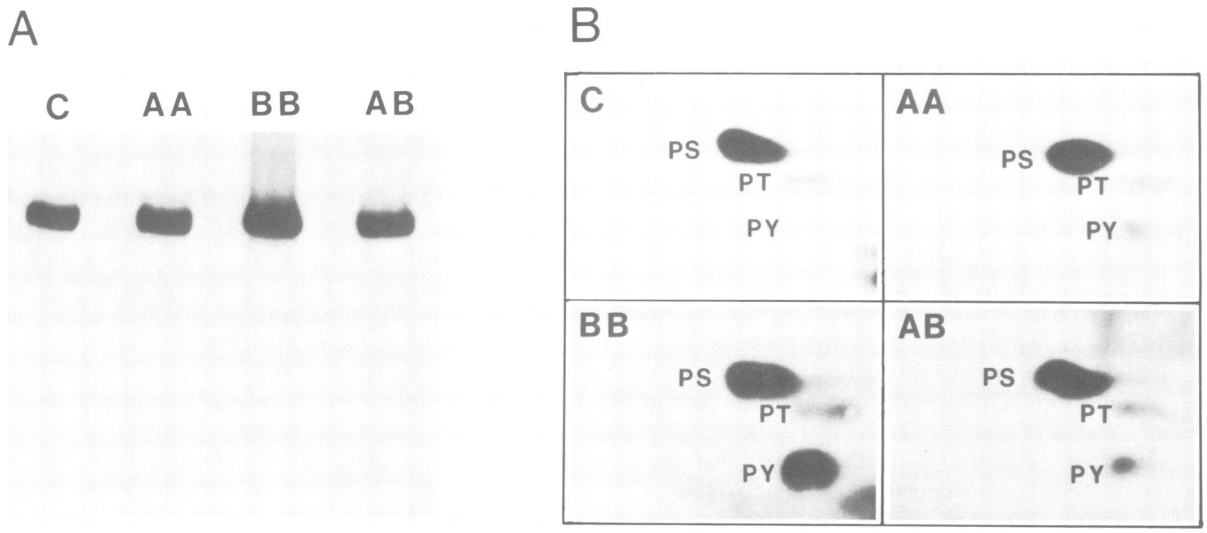

Fig. 1. Antireceptor immunoprecipitates of AG1523 fibroblasts incubated with PDGF dimers. Dishes (9-cm) of confluent, quiescent AG1523 human fibroblasts were labeled with 1 mCi/ml $^{32}P_i$ for 4 h, then incubated without PDGF (C) or with 10 ng/ml AA, 10 ng/ml AB, or 50 ng/ml BB PDGF for an additional 10 min at 37°C. (**A**) Cells were lysed, immunoprecipitated with a monoclonal antibody to the PDGF receptor and resolved on an SDS–polyacrylamide gel (7.5% acrylamide, 0.195% bisacrylamide). The receptor band was excised and quantitated by Cerenkov counting: C, 4902 c.p.m.; AA, 5334 c.p.m.; BB, 10 461 c.p.m.; AB, 5542 c.p.m. (**B**) The phosphoamino acid composition was determined according to Cooper *et al.* (1983b). PS, phosphoserine; PT, phosphothreonine; PY, phosphotyrosine.

with unlabeled AA. AA was able to block 4−5% of subsequent BB binding to AG1523 or SK-5 cells (Seifert *et al.*, submitted). BB binding sites were also shown to outnumber AA binding sites on SK-5 human fibroblasts by down-regulation with AA PDGF (Hart *et al.*, 1988). In contrast, in Swiss 3T3 cells, competition experiments suggested that AA binding sites were 45% as abundant as BB binding sites (Seifert *et al.*, submitted). Thus in AG1523 and SK-5 cells the poor mitogenic response to the AA dimer may be due to a much lower number of AA binding sites on these cell types.

Receptor phosphorylation stimulated by the three forms of PDGF

Phosphorylation of the receptor on tyrosine is one of the earliest known responses to PDGF, and is thought to initiate many of the subsequent PDGF-dependent events. $^{32}P_i$-labeled cells were incubated with each ligand for 10 min, lysed and immunoprecipitated with either monoclonal antibody PR7212, which recognizes an extracellular epitope β subunit of the receptor (Hart *et al.*, 1988; Seifert *et al.*, submitted), or with monoclonal antiphosphotyrosine antibody 1G2 (Frackelton *et al.*, 1983). The former antibody could only be used with human cells (Hart *et al.*, 1987).

Immunoprecipitation of PDGF receptors from unstimulated AG1523 cells with PR7212 showed that the receptor is phosphorylated basally on serine and, to a trace extent, threonine (Figure 1). Stimulation with any PDGF dimer caused little change in phosphoserine or phosphothreonine content, but increased receptor phosphorylation at tyrosine. Stimulation with BB PDGF increased total receptor phosphorylation by ~100%, with most of the increase due to extensive phosphorylation at tyrosine (Figure 1). Stimulation with AA or AB caused little change in total phosphate content, but phosphorylation of tyrosine was detectable, at ≤10% of the level stimulated by BB (Figure 1).

Antiphosphotyrosine antibody immunoprecipitated a number of proteins from PDGF-stimulated Swiss 3T3 cells that were not precipitated from control cells (Figure 2). The receptor spot (Figure 2, arrowhead) was identified by

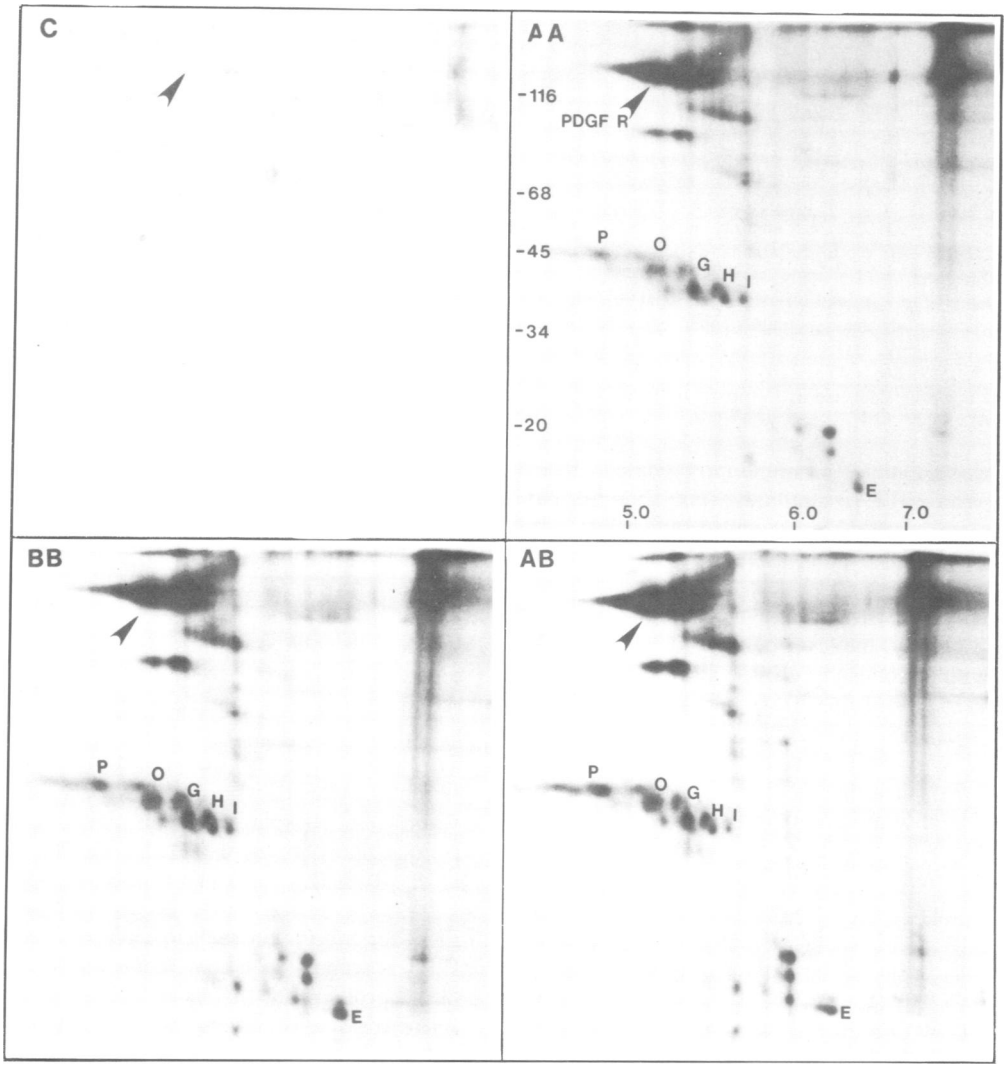

Fig. 2. Antiphosphotyrosine immunoprecipitates of Swiss 3T3 cells incubated with PDGF dimers. Confluent, quiescent Swiss 3T3 cells in 9-cm dishes were labeled and stimulated with PDGF as in Figure 1. Cells were lysed and immunoprecipitated with antiphosphotyrosine antibody 1G2 as described in Materials and methods. Immune complexes were dissociated competitively in the presence of 1 mM phenyl phosphate, and resolved by two-dimensional gel electrophoresis. Acidic proteins are to the left. Gels were dried and subjected to autoradiography at −70°C with intensifying screens. The arrowhead points to the position of the PDGF receptor; P, O, G, H, I, E denote PDGF-dependent phosphoproteins which contain phosphotyrosine (Kazlauskas and Cooper, 1988).

Table II. Protein phosphorylation stimulated by different forms of PDGF

Cell type	PDGF	PDGF receptor[a]	pp36[b]	pp42A[c]
AG1523	C	0	1	24
	AA	2	11	107
	AB	28	26	103
	BB	100	100	100
SK-5	C	2	2	64
	AA	25	9	118
	AB	38	38	114
	BB	100	100	100
3T3	C	0	4	24
	AA	45	5	149
	AB	94	69	112
	BB	100	100	100

$^{32}P_i$-labeled cells were incubated for 10 min with no additions (C), 10 ng/ml AB, 10 ng/ml AA or 50 ng/ml BB PDGF. Results are a compilation of separate experiments, in which cellular proteins were either analyzed by two-dimensional gel electrophoresis or immunoprecipitated with antiphosphotyrosine antibodies. Incorporation of radioactivity into different proteins is presented as a percentage of incorporation in cells stimulated with BB.

[a]Samples of antiphosphotyrosine immunoprecipitates were subjected to one- (SK-5) or two- (AG1523 and 3T3) dimensional gel electrophoresis. Receptor phosphorylation was estimated either by densitometry of autoradiograms (AG1523 and SK-5 cells), using the Visage 110 system (BioImage, Ann Arbor, MI), or by excising the part of the gel containing the receptor and Cerenkov counting (3T3 cells); 100% values were: AG1523, 74.5 intensity units; SK-5, 4.98 intensity units; Swiss 3T3, 482 c.p.m. Substantially similar results were obtained for SK-5 cells by Cerenkov counting.

[b]Total phosphoproteins were separated on two-dimensional gels that were incubated in alkali before autoradiography. pp36 phosphorylation was estimated by densitometry; 100% values were: AG1523, 14.25 intensity units; SK-5, 29.9; Swiss 3T3, 2.7.

[c]pp42A phosphorylation was estimated as for pp36; 100% values were: AG1523, 8.61; SK-5, 14.99; Swiss 3T3, 2.36.

co-migration with material immunoprecipitated from AG1523 cells with PR7212 antibody (not shown). No receptor band was detectable in untreated cells. AA stimulated ~45% of the receptor phosphorylation stimulated by either AB or BB in Swiss 3T3 cells (Figure 2 and Table II). In AG1523 fibroblasts, AA and AB PDGF stimulated 2 and 28%, respectively, the level of receptor phosphorylation, detected with antiphosphotyrosine antibody, that was stimulated by BB PDGF (Table II). In human SK-5 dermal fibroblasts, AA was 25% and AB was 38% as effective as BB at stimulating receptor tyrosine phosphorylation (Table II). In all cases, phosphoamino acid analysis of the PDGF receptor precipitated with antiphosphotyrosine antibody revealed phosphotyrosine and phosphoserine in approximately equimolar amounts (Kazlauskas and Cooper, 1988).

Phosphate esterified to tyrosine residues is more resistant to alkali than that on serine residues (Cheng and Chen, 1981; Cooper and Hunter, 1981). Thus the phosphotyrosine content of PDGF receptors may be estimated by quantitation of its alkali-stable phosphate, following separation of the receptor from other phosphoproteins by two-dimensional gel electrophoresis (e.g. see Figure 5). In numerous experiments, the level of alkali-stable receptor phosphorylation was low for AA or AB PDGF in human cells, but similar for AA, AB and BB PDGF in Swiss 3T3 cells (not shown).

All three methods of analysis suggested that all forms of PDGF can stimulate receptor phosphorylation on tyrosine. The labeling of tyrosine-phosphorylated receptors was proportional, to within experimental error, to the numbers of binding sites for each ligand (Table II and Seifert *et al.*, submitted).

Phosphorylation of other cell proteins at tyrosine

Antiphosphotyrosine immunoprecipitates of PDGF-treated $^{32}P_i$-labeled Swiss 3T3 cells contained a number of other phosphoproteins, including P, O, G, H, I and E (Figure 2) which each contained phosphotyrosine (Kazlauskas and Cooper, 1988). The patterns of these phosphoproteins were qualitatively similar in Swiss 3T3 or AG1523 cells treated with each of the PDGF dimers (Figure 2 and data not shown).

Certain phosphotyrosine-containing proteins are precipitated poorly by antiphosphotyrosine antibodies, but are phosphorylated sufficiently to be detected when total cellular phosphoproteins are separated by two-dimensional gel electrophoresis and the gels are incubated in alkali prior to autoradiography (Kazlauskas and Cooper, 1988). One of the proteins which is phosphorylated on tyrosine in response to PDGF or epidermal growth factor is p36, also known as calpactin I or lipocortin II (Cooper *et al.*, 1982). p36 is a peripheral membrane protein and a substrate for growth factor receptor kinases *in vitro* (reviewed in Cooper and Hunter, 1983), suggesting that receptors may phosphorylate p36 directly in living cells. p36 was barely phosphorylated in unstimulated AG1523 fibroblasts (Figure 3). BB stimulated the greatest incorporation of phosphate into p36, AB an intermediate amount, and AA stimulated the least incorporation into p36 (Figure 3). Similar p36 phosphorylation results were obtained in two other experiments with AG1523 human fibroblasts, as well as with SK-5 human fibroblasts (Table II and data not shown). The low extent of p36 phosphorylation stimulated by AA in these cell types could be due to the low number of AA binding sites.

Swiss 3T3 cells bind one-half as much AA PDGF as AB or BB PDGF. In these cells, the extent of p36 phosphorylation was similar when cells were incubated with either AB or BB, but was barely above the basal level when AA was used (Table II and Figure 4A). The reduced phosphorylation of p36 in response to the AA dimer in Swiss 3T3 cells suggests that the level of p36 phosphorylation may not be directly proportional to receptor occupancy, but instead require some minimum level of protein tyrosine kinase activity to be achieved in order for it to be phosphorylated. This would resemble the phosphorylation of p36 in chick fibroblasts infected with various Rous sarcoma virus mutants, where p36 phosphorylation is only increased when total cellular phosphotyrosine levels exceed a threshold (Cooper *et al.*, 1983a).

PKC-dependent protein phosphorylation events

PKC is stimulated when PDGF binds to its receptor. An 80 kd protein (p80) is rapidly phosphorylated on multiple serine residues following PKC activation (Rozengurt *et al.*, 1983; Albert *et al.*, 1986; Blackshear *et al.*, 1986; Isacke *et al.*, 1986). We compared the ability of the three PDGF dimers to induce p80 phosphorylation. $^{32}P_i$-labeled Swiss 3T3 cells were incubated with the various dimers for 10 min, then lysed and analyzed by two-dimensional gel electrophoresis. Phosphoproteins were detected by autoradiography

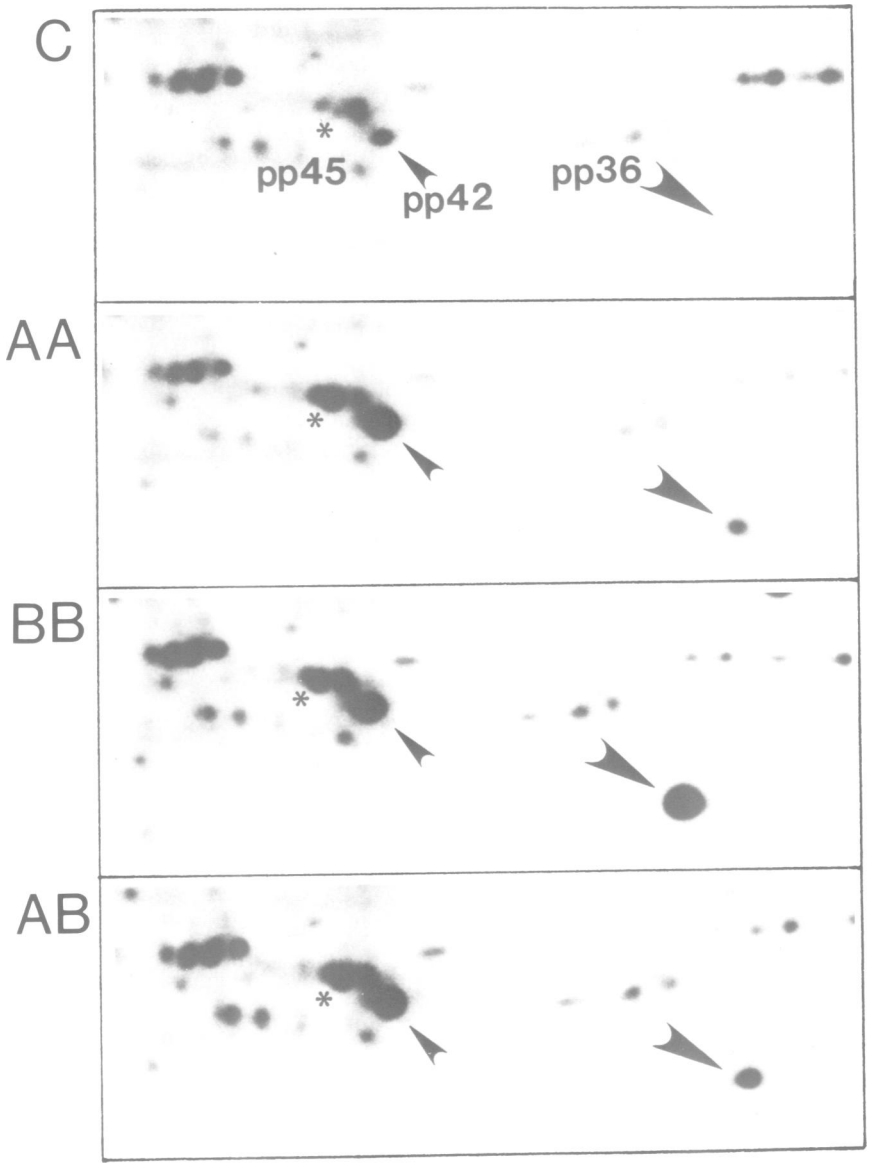

Fig. 3. Phosphorylation of p45, p42 and p36 in AG1523 fibroblasts stimulated with dimers of PDGF. Dishes (35-mm) of quiescent AG1523 human fibroblasts were labeled and stimulated with PDGF as described in Figure 1. Cells were lysed and total phosphoproteins were analyzed by two-dimensional electrophoresis as described in Materials and methods. Portions of autoradiograms of alkali-treated gels are shown. An asterisk marks the position of pp45B; the small arrowhead pointing upward denotes pp42A, while the location of pp36 is identified by the large arrowhead pointing downward.

of gels that had not been incubated in alkali. All ligands induced a similar level of p80 phosphorylation in Swiss 3T3 cells (Figure 4B, arrowhead). In AG1523 and SK-5 cells, basal p80 phosphorylation was high, and it was not possible to determine whether PDGF stimulated further phosphorylation (not shown). However, when AG1523 cells were incubated with or without PDGF for 1 h at 4°C, instead of for 10 min at 37°C, basal p80 phosphorylation was greatly reduced, and all three dimers strongly stimulated p80 phosphorylation (not shown). Thus, unlike the phosphorylation of p36 or PDGF receptor, it appears that all three PDGF dimers were able to equally activate PKC in both human fibroblasts and mouse 3T3 cells.

We also compared the ability of the three dimers to activate PKC by examining additional PKC-dependent events. Two scarce cytoplasmic proteins, p42 and p45, are phosphorylated on tyrosine and serine when PKC is activated by PDGF treatment (Cooper *et al.*, 1982; Kazlauskas and Cooper, 1988). All PDGF dimers stimulated p42 and p45 phosphorylation to a comparable extent in AG1523 fibroblasts (Figure 3 and Table II) and in Swiss 3T3 cells (Figure 4A and Table II). Similar results were observed in replicate experiments with AG1523 and 3T3 cells, as well as with SK-5 cells (Table II and data not shown). These results suggest that saturating doses of all PDGF dimers activate PKC equally, independent of cell type.

Effects of low occupancy of BB binding sites

Since AA binds to only 5% of the sites available to BB in AG1523 cells, but maximally stimulates PKC-dependent serine and tyrosine phosphorylations (Figure 3 and Table II), it appears that PKC is activated by the occupation of only a small fraction of the total receptor population. This leads to the prediction that a submaximal dose of BB could

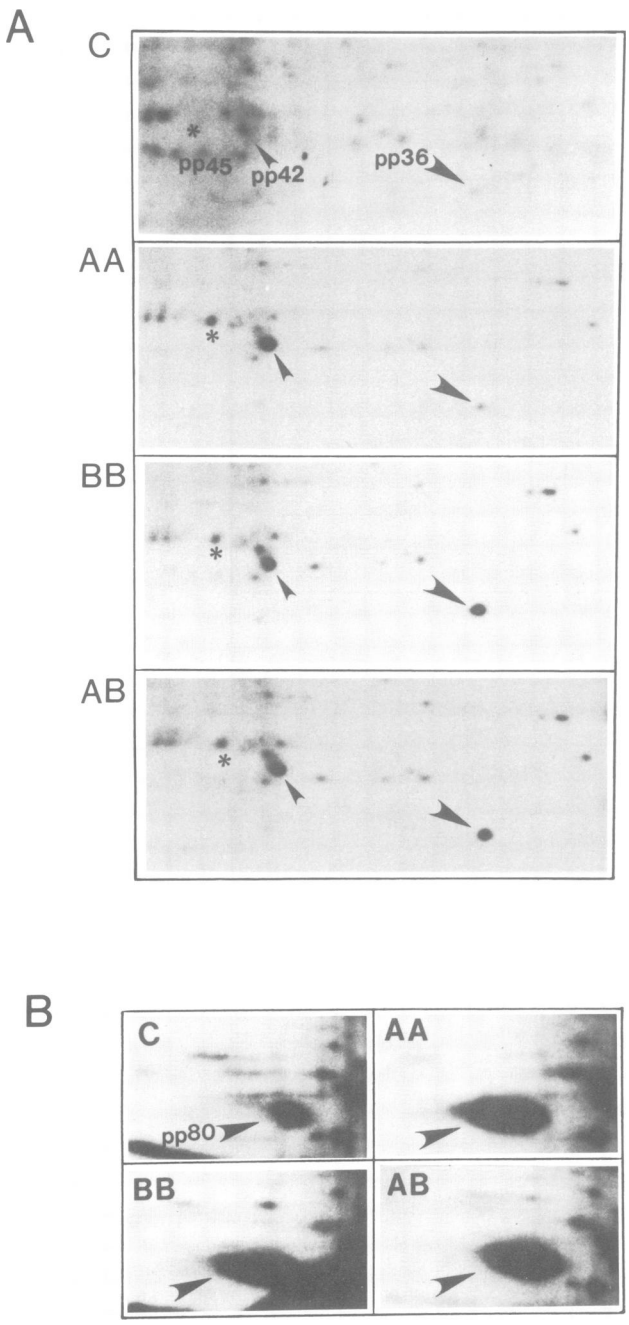

Fig. 4. Phosphorylation of p80, p45, p42 and p36 in Swiss 3T3 cells stimulated with PDGF dimers. Quiescent Swiss 3T3 cells in 35-mm dishes were labeled and stimulated with PDGF as described in Figure 1. Total phosphoproteins were resolved by two-dimensional electrophoresis as described in Materials and methods. (**A**) Portions of autoradiograms of alkali-treated gels are shown. An asterisk marks the position of pp45B, the small arrowhead pointing upward denotes pp42A, while the location of pp36 is identified by the large arrowhead pointing downward. Panel C was exposed twice as long to demonstrate the absence of pp42A, pp45B and pp36. (**B**) A portion of the autoradiograms from non-alkali-treated gels containing the pp80 region is shown. The arrowhead points to pp80.

be as effective as a maximal one in initiating PKC-dependent events. To test this possibility, $^{32}P_i$-labeled Swiss 3T3 cells were incubated with either 0, 2.5 or 50 ng/ml of the BB dimer for 10 min, and their phosphoproteins analyzed. Measurement of ^{125}I-labeled BB binding under identical conditions showed that only 17% as much BB PDGF bound to Swiss 3T3 cells when added at 2.5 ng/ml concentration as bound at 50 ng/ml concentration (data not shown). When total phosphoproteins were analyzed, receptor phosphorylation was only detected with the high dose (Figure 5, small arrowhead in the left panel), but could be detected with both doses of BB when antiphosphotyrosine immunoprecipitates were analyzed (Table III). Similarly, both doses of PDGF stimulated p36 phosphorylation (Figure 5, right panel), and the high dose was 14 times more effective (Table III). In contrast, we found that the limiting dose of PDGF initiated ~50% of maximal p80 phosphorylation (Figure 5, left panel, large arrowhead), and maximal p42 and p45 phosphorylation (Figure 5, right panel and Table III). Thus it appears that occupancy of only a small fraction of the total pool of PDGF receptors is needed to activate PKC and subsequent PKC-dependent events.

Discussion

The mitogenic effects of the three PDGF dimers differed according to the cell type studied. Two different lines of human fibroblasts were poorly stimulated by AA, while AB and BB stimulated a strong mitogenic response. Swiss 3T3 cells were similarly sensitive to all three forms of PDGF. Therefore the low potency of AA in human cells is not due to inactivation of this molecule during its preparation from yeast cells. Instead, it appears that the weaker mitogenic effects of AA for human cells are due to the relatively low number of type A/B binding sites (Nister *et al.*, 1987; Hart *et al.*, 1988; Seifert *et al.*, submitted). The phosphorylation events we studied fall into a hierarchy according to the dependence on dose and type of ligand. Activation of PKC to high levels requires occupancy of only a low number of PDGF receptors (Figures 3–5 and Tables II and III). Tyrosine phosphorylation of the receptor, and of a number of other cell proteins (Figure 2, Tables II and III), is more directly proportional to the number of occupied binding sites, and therefore differs according to the form of PDGF and the cell type. Finally, phosphorylation of p36 in Swiss 3T3 cells may require receptor kinase activity to exceed a threshold level (Figure 4, Table II), much as its phosphorylation in chick cells by the protein-tyrosine kinase encoded by Rous sarcoma virus requires high levels of kinase activity (Cooper *et al.*, 1983a).

Nister *et al.* (1988) reported that AA stimulated receptor phosphorylation and DNA synthesis poorly in AG1523 fibroblasts, when compared with human platelet PDGF (chiefly AB). Our data suggest that the reduced ability of AA to stimulate receptor tyrosine phosphorylation and mitogenesis in AG1523 cells and SK-5 cells may be largely due to the reduced number of α receptor subunits which are necessary for binding of AA. In Swiss 3T3 cells, which have similar numbers of α and β receptor subunits, all dimers bind to a comparable extent, and were similarly effective in inducing receptor phosphorylation and DNA synthesis. Although competition, down-regulation and cDNA experiments suggest multiple types of PDGF binding sites, our data suggest that binding to the different types of sites triggers similar cellular responses. Differences between different forms of PDGF seem to be essentially quantitative in nature.

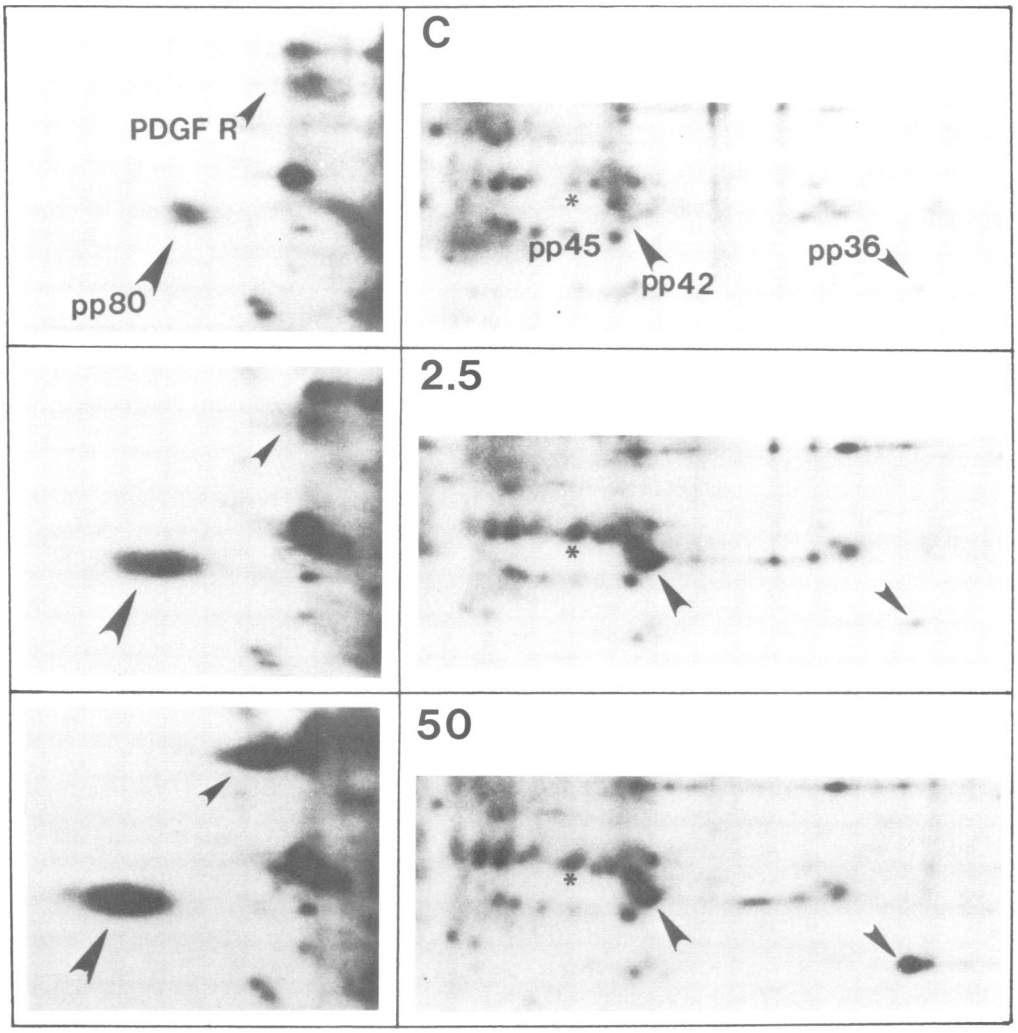

Fig. 5. Stimulation of Swiss 3T3 cells with a submaximal dose of PDGF initiates a maximal PKC-dependent response. Dishes (35-mm) of confluent, quiescent Swiss 3T3 cells were labeled and stimulated as in Figure 1, except the concentration of the BB dimer was either 2.5 or 50 ng/ml. Cells were lysed and total phosphoproteins were analyzed by two-dimensional electrophoresis as described in Materials and methods. Gels were alkali-treated before autoradiography. The gel of control cells (C) was exposed for approximately twice as long as the other gels to obtain an exposure of similar intensity. **Left-hand panels**: large arrowhead points to pp80 and a small arrowhead marks the position of the PDGF receptor. **Right-hand panel:** the arrowhead pointing upward denotes the position of pp42A, the arrowhead pointing downward marks the position of pp36 and the asterisk indicates the position of pp42B.

Our data suggest that the α and β subunits of the PDGF receptor have similar biochemical acivities.

All dimers triggered PKC-dependent phosphorylations equally well. In AG1523 and SK-5 cells, the AA dimer appeared to strongly activate PKC despite binding to only 4−5% of the sites available to BB dimers. This did not appear to be unique to the AA dimer, since a submaximal concentration of BB, sufficient to mediate the phosphorylation of only 8% of the total receptor population in Swiss 3T3 cells (Table III), was also able to initiate near maximal phosphorylation of PKC substrates. These observations predict that no more than 5−10% of the total population of PDGF receptors need to be phosphorylated to initiate phosphotidylinositol turnover. In addition, since PKC activation and subsequent PKC-dependent events do not correlate with mitogenesis in human fibroblasts (Table I), it seems unlikely that these events are sufficient to elicit a maximal mitogenic response. A similar conclusion has been reached from the observation that platelet PDGF is mitogenic for cells in which PKC has been depleted by chronic exposure to PKC activators (Coughlin *et al.*, 1985). Furthermore, our results suggest that extensive phosphorylation of p36 (calpactin I or lipocortin II) is not necessary for mitogenesis, because it is phosphorylated poorly in response to AA dimers in Swiss 3T3 cells, in which cells AA dimers are fully mitogenic (Figure 4, Table I).

Phosphoamino acid analysis of the PDGF receptor from $^{32}P_i$-labeled cells showed no changes in the phosphoserine and phosphothreonine content within the first 10 min of ligand binding. The epidermal growth factor receptor is phosphorylated by PKC and other serine kinases in response to the binding of epidermal growth factor as well as a number of other mitogens, including PDGF (Davis and Czech, 1984, 1987; King and Cooper, 1986). There has been no evidence to date that the PDGF receptor is phosphorylated by PKC, and its phosphorylation state does not change dramatically

Table III. Protein phosphorylation in Swiss 3T3 cells stimulated with different doses of BB PDGF

BB (ng/ml)	PDGF receptor[a]	pp36[b]	pp42A[b]
0	1	3	12
2.5	9	7	140
50.0	100	100	100

Confluent, quiescent Swiss 3T3 cells in 60-cm dishes were labeled with 1 mCi/ml $^{32}P_i$ for 4 h and then incubated with the indicated concentration of the BB dimer for an additional 10 min. Results are a compilation of separate experiments, in which cellular proteins were either analyzed by two-dimensional gel electrophoresis or immunoprecipitated with antiphosphotyrosine antibodies. In separate experiments, cells were either analyzed by two-dimensional gel electrophoresis or immunoprecipitated with an antiphosphotyrosine antibody. Incorporation of radioactivity into different proteins is expressed as percentage of incorporation with 50 ng/ml BB.
[a]Immunoprecipitated proteins were resolved on an SDS–polyacrylamide gel (7.5% acrylamide, 0.195% bisacrylamide). The radiolabeled 180 kd region was localized by autoradiography, excised and quantitated by Cerenkov counting; 100%, 2798 c.p.m.
[b]pp36 and pp42A were estimated by two-dimensional densitometry of autoradiograms of alkali-treated two-dimensional gels; 100% values were: pp36, 4.05 intensity units; pp42A, 5.09 intensity units.

in cells made PKC deficient (A.Kazlauskas and J.A.Cooper, unpublished observations). While PDGF activates a number of serine/threonine-specific kinases including PKC (Habenicht *et al.*, 1981; Isacke *et al.*, 1986) and the S6 kinase (Nishimura and Deuel, 1983; Wettenhall *et al.*, 1983), the kinases that phosphorylate the receptor on serine and threonine were not affected within the first 10 min of PDGF stimulation. The identity of the serine/threonine kinase(s) which phosphorylates the PDGF receptor in living cells, and the consequences of this modification, remain unresolved.

Recent reports have suggested that binding sites for different PDGF dimers may have distinct structures. Two groups have observed that the three forms of PDGF do not equally compete one another's binding, and that monoclonal antibodies to the PDGF receptor recognize only one type of PDGF binding site or subunit (Hart *et al.*, 1988; Heldin *et al.*, 1988). By affinity cross-linking radiolabeled PDGF dimers to cells, Heldin *et al.* (1988) detected two different PDGF receptor species: AA bound to a 140 kd species, BB to a 175–190 kd complex, while AB bound to both of these species. Gronwald *et al.* (1988) and Claesson-Welsh *et al.* (1988) expressed human PDGF receptor cDNAs in BHK or CHO cells, respectively, and observed the high affinity binding of only the BB dimer, suggesting that these cDNAs encode the β subunit. In contrast, Escobedo *et al.* (1988) expressed a human PDGF receptor cDNA in CHO cells, and found that saturating concentrations of each PDGF dimer had equivalent effects with respect to receptor phosphorylation and mitogenesis.

We have observed that all PDGF dimers stimulate tyrosine phosphorylation of a receptor species (Figures 1 and 2). If tyrosine phosphorylation of PDGF receptor subunits is intramolecular, this result suggests that both the α and β subunits are tyrosine kinases that become phosphorylated in response to ligand binding. In addition, the mol. wt and isoelectric point (Figure 2), as well as the phosphopeptide maps, of the α and β subunits are indistinguishable (Kazlauskas and Cooper, unpublished results). If the two receptors are encoded by distinct genes, it seems likely that the gene products are strikingly similar.

Materials and methods

Materials

Human A- [endothelial, or short, form (Collins *et al.*, 1987a; Tong *et al.*, 1987)] and B-chains were independently expressed in the yeast *Saccharomyces cerevisiae*. The two homodimers were purified to >95% homogeneity, assessed by amino-terminal sequencing and by SDS–PAGE under reducing or non-reducing conditions (Hart *et al.*, 1988). Unpurified yeast media containing the secreted, expressed proteins were used in some of the experiments. Crude and purified preparations of AA and BB behaved identically. The AB heterodimer was purified from human platelets as described previously (Hart *et al.*, 1988) to ≥95% homogeneity (analyzed by SDS–PAGE and amino-terminal sequencing). The concentrations of PDGF refer to protein concentrations determined by amino acid analysis.

Cell culture

Swiss 3T3 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% calf serum (CS). SK-5 human dermal fibroblasts and AG1523 human neonatal foreskin fibroblasts were maintained in DMEM with 10% fetal bovine serum.

Metabolic labeling of cells

Confluent, quiescent cultures of cells were rinsed twice in H/S buffer (20 mM Hepes, pH 7.4, 150 mM NaCl) and incubated overnight in labeling medium (DMEM containing 10% of the normal phosphate concentration, 15 mM Hepes, pH 7.4, 0.1% CS). $^{32}P_i$ was added to a final concentration of 1 mCi/ml (unless indicated otherwise) and the incubation at 37°C was continued for 4 h. PDGF was added during the last 10 min of the 4 h incubation, after which the cells were placed on ice and washed twice with ice-cold H/S buffer. Samples were prepared for two-dimensional electrophoresis as previously described (Kazlauskas and Cooper, 1988) or for immunoprecipitation as described below.

Two-dimensional gel electrophoresis

Samples were separated in the first dimension by isoelectric focusing, using pH 3.5–10 range ampholytes, followed by SDS–PAGE (15% acrylamide, 0.086% bisacrylamide) in the second dimension, as previously described (Garrels, 1979; Cooper and Hunter, 1981). Acidic proteins focused to the left of the gels. Samples were normalized on the basis of cell number, and lysate equivalent to 2×10^4 cells were loaded per gel. For alkali treatment (Cheng and Chen, 1981; Cooper and Hunter, 1981), dried gels were rehydrated, incubated in 1 M KOH for 2 h at 56°C, fixed in 10% acetic acid and 10–20% isopropanol, and dried under vacuum. All gels were exposed with intensifying screens at −70°C.

Immunoprecipitation with antiphosphotyrosine antibody

Confluent, quiescent, 90-mm dishes of cells were metabolically labeled with 1 mCi/ml $^{32}P_i$ and stimulated with PDGF as described above. Dishes of cells were placed on ice and quickly cooled by washing twice with H/S buffer. Cells were lysed and immunoprecipitated as previously described (Frackelton *et al.*, 1983; Kazlauskas and Cooper, 1988).

Immunoprecipitation with antibody to the PDGF receptor

Cells were washed, lysed, vortexed and centrifuged as described above for immunoprecipitation with the antiphosphotyrosine antibody, except that the lysis buffer was RIPA [0.15 M NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% sodium dodecyl sulphate (SDS), 10 mM sodium phosphate, pH 7.0, 2 mM EDTA, 14 mM 2-mercaptoethanol, 20 μg of aprotinin/ml, 50 mM NaF, 2 mM Na_3VO_4]. The supernatant from the centrifugation step was incubated for 5 min at 0°C with 10 mg fixed *Staphylococcus aureus* (Pansorbin, Calbiochem, La Jolla, CA), centrifuged for 5 min at 8000 *g*, and the supernatant was transferred to a new tube containing 20 μg of monoclonal anti-PDGF receptor antibody PR7212 (Hart *et al.*, 1987). After 2 h on ice, 68 μg of a polyclonal rabbit anti-mouse secondary antibody was added, incubated for an additional 60 min at 0°C, at which time 7 mg of fixed *S.aureus* was added and incubated for 30 min on ice. The samples were layered over 600 μl of RIPA supplemented with 10% sucrose and centrifuged for 20 min at 1500 *g* in a JS 5.2 swinging-bucket rotor. The immune complexes were washed twice with RIPA and twice with a buffer containing 0.5% NP-40, 0.1 M NaCl, 10 mM Pipes, pH 7.0, 1% aprotinin. Immune complexes were dissociated by adding sample buffer (2% SDS, 2.5 mM EDTA, 2.8 M 2-mercaptoethanol, 10% glycerol, 100 mM Tris, pH 6.8, 0.01% bromophenol blue) and heating to 95°C for 3–5 min.

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