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Increased mitogenicity of an $\alpha\beta$ heterodimeric PDGF receptor complex correlates with lack of RasGAP binding

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The different platelet-derived growth factor (PDGF) isoforms cause activation of their α and β protein tyrosine kinase receptors through dimerization. Homodimerization as well as heterodimerization of receptors occur. It has been shown previously that the heterodimeric receptor complex mediates a stronger mitogenic response than either of the homodimeric complexes. In this report, we show that in cells expressing both PDGF α- and β-receptors, stimulation with PDGF-AB, which leads to preferential heterodimer formation, leads to a very low degree of phosphorylation of Tyr771 in the β-receptor. In contrast, Tyr771 is phosphorylated in a homodimeric complex of β-receptors. Phosphorylated Tyr771 is a binding site for RasGAP; an analogous site is not present in the α-receptor, which lacks the ability to associate with RasGAP. The lowered phosphorylation of Tyr771 in the heterodimeric receptor complex correlates with lowered association with RasGAP, as well as with a more efficient activation of Ras and MAP kinase, which is consistent with the increased mitogenicity elicited by PDGF-AB, compared to PDGF-AA or PDGF-BB.

Keywords: heterodimer; mitogenicity; PDGF receptor; phosphorylation; RasGAP

Introduction

Receptor tyrosine kinases are often activated by ligand-induced dimerization (reviewed in Heldin, 1995). This leads to autophosphorylation of tyrosine residues in the intracellular parts of the receptors, which then act as docking sites for down-stream signal transduction molecules containing SH2 or PTB domains (reviewed in Pawson and Scott, 1997). Since the amino acid sequence around the phosphorylated tyrosine determines the specificity for binding of individual SH2 and PTB domains, the exact localization of phosphorylated tyrosine residues will determine which signals are induced.

In addition to homodimerization of tyrosine kinase receptors, specific heterodimeric receptor complexes of structurally related receptors can also be formed after ligand binding. Examples include heteromeric complexes of members of the epidermal growth factor receptor family (Carraway and Cantley, 1994; Soltoff *et al*., 1994; Wada *et al*., 1989; Heidaran *et al*., 1991; Kanakaraj *et al*., 1991; Seifert *et al*., 1989). PDGF is a family of dimeric isoforms of related A- and B-polypeptide chains, which exert their cellular effects by binding to two structurally similar tyrosine kinase receptors. Since the B-chain binds to both α- and β-receptors with high affinity, whereas the A-chain binds only to α-receptors, the different PDGF isoforms induce different dimeric receptor complexes; PDGF-AA induces $\alpha\alpha$ receptor dimers, PDGF-AB $\alpha\alpha$ and $\alpha\beta$ receptor dimers and PDGF-BB all three possible receptor combinations.

Homodimerization of PDGF α- or β-receptors has been shown to lead to autophosphorylation of at least five or ten tyrosine residues, respectively (reviewed in Heldin *et al*., 1998) which interact in a specific manner with several SH2 domain containing signal transduction molecules. Phosphatidylinositol-3′-kinase, phospholipase C-γ, and the cytoplasmic tyrosine kinases of the Src family are examples of signal transduction molecules binding to both α- and β-receptors, whereas the GTPase activating protein (GAP) of Ras binds only to β-receptors (Bazenet and Kazlauskas, 1994; Heidaran *et al*., 1993) and the adaptor molecule Crk only to α-receptors (Yokote *et al*., 1998).

Among the PDGF isoforms, PDGF-AB has been reported to have the most potent mitogenic effect on cells expressing both α- and β-receptors (Heidaran *et al*., 1991; Rupp *et al*., 1994). We therefore investigated whether the heterodimeric PDGF receptor complex has unique properties compared to the homomeric receptor complexes. We report that the heteromeric complex gives a stronger and more sustained activation of Ras and Erk2 compared to homodimeric complexes, which may be explained by our finding that RasGAP, which deactivates Ras, binds to the $\beta\beta$ receptor homodimer, but not to the $\alpha\beta$ receptor heterodimer. The mechanism behind this difference appears to involve autophosphorylation of Tyr771, to which RasGAP binds, in the β-receptor in the homodimer, but not in the β-receptor in the heterodimeric complex.

Results

Efficient mitogenic stimulation of PAE cells expressing both PDGF α- and β-receptors by PDGF-AB

PAE cells, which lack endogenous PDGF receptors, were used as recipient cells for the establishment of cell lines stably expressing both α- and β-receptors at equal levels. Three different PAE/$\alpha\beta$ cell lines expressing approximately 50 000 each of α- and β-receptors, as determined by Scatchard analyses (data not shown), were selected for further studies.

The abilities of the different PDGF isoforms to stimulate incorporation of [^{3}H]thymidine in PAE/$\alpha\beta$

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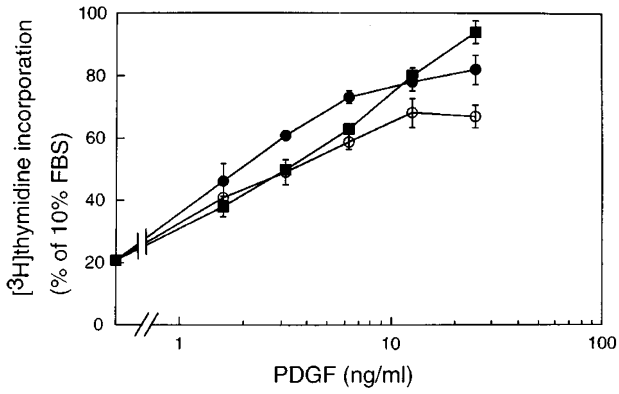

Figure 1 Effect of PDGF isoforms on mitogenicity of PAE/$\alpha\beta$ cells. After 24 h of starvation in 1 mg/ml BSA, PAE/$\alpha\beta$ cells were incubated with [^{3}H]thymidine at the indicated concentrations of PDGF-AA (○), PDGF-AB (●) or PDGF-BB (■) for 24 h at 37°C. Incubation with 10% FBS was used as a positive control. After incubation, trichloroacetic acid-precipitated [^{3}H]thymidine radioactivity was measured. Data represent mean values of triplicates with indicated standard deviation and are expressed as percentage of the value obtained for stimulation with 10% FBS. Shown is a representative result; the experiment was repeated four times

cells were investigated. As shown in Figure 1, PDGF-AB gave a higher incorporation of [^{3}H]thymidine than PDGF-AA or PDGF-BB at concentrations up to 10 ng/ml. The differences are rather small but reproducible and consistent with previous observations (Rupp *et al*., 1994).

PDGF-AB induces more efficient activation of Ras and MAP kinase than other PDGF isoforms

Activation of Ras and subsequent activation of the kinases in the MAP kinase cascade, i.e. Raf-1, MEK and MAP kinase, has been shown to be important for mitogenic stimulation (Cai *et al*., 1990; Mulcahy *et al*., 1985). PDGF-stimulation has been shown to cause activation of Ras via direct or indirect binding of the nucleotide exchange protein Sos1 in complex with the adaptor Grb2 to the PDGF receptors (Arvidsson *et al*., 1994; Li *et al*., 1993; Yokote *et al*., 1994). We therefore investigated the efficiencies of various PDGF isoforms to activate Ras and MAP kinase.

To estimate Ras activation, ^{32}P-orthophosphate labeled PAE/$\alpha\beta$ cells were stimulated with different PDGF isoforms, followed by immunoprecipitation of Ras; the binding of GTP and GDP to Ras, as markers of active and inactive Ras, respectively, was determined by thin layer chromatography. As seen in Figure 2, all PDGF isoforms increased the GTP/GDP ratio on Ras, with a maximum after 5 min. PDGF-AB reproducibly gave a slightly higher activation of Ras compared to the other isoforms.

One of the downstream effectors of Ras is Raf-1, the first kinase in the MAP kinase cascade. MAP kinase activity was measured after different time periods of stimulation by the different PDGF isoforms, using myelin basic protein as a substrate. In this assay, a biphasic response was obtained with an early peak at about 5 min and a later after 60 min; during both these phases PDGF-AB gave higher activation than PDGF-AA or PDGF-BB (Figure 3).

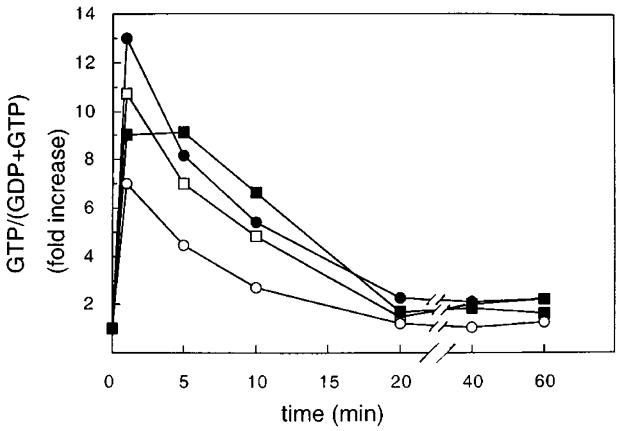

Figure 2 Increased Ras GTP loading by stimulation with PDGF isoforms. PAE/$\alpha\beta$ cells were subjected to *in vivo* ^{32}P-orthophosphate labeling for 4.5 h at 37°C and then stimulated with PDGF-AA (□), PDGF-AB (●), or PDGF-BB (■), or left unstimulated for indicated time periods at 37°C. PAE/PDGFRβ cells were analysed in parallel and stimulated with PDGF-BB (○) or left unstimulated. Cells were lysed and the lysates incubated with Ras antibodies. Immunoprecipitated Ras was eluted from Protein A-Sepharose beads and separated by chromatography on polyethyleneimine (PEI)-cellulose plates. The spots corresponding to radioactive GDP and GTP were quantified using a Fuji BAS 2000 Bioimage Analyzer and the GTP/(GDP+GTP) ratio was calculated. Data are from a representative experiment of four experiments performed, and presented as fold increased compared to control, where no ligand was added

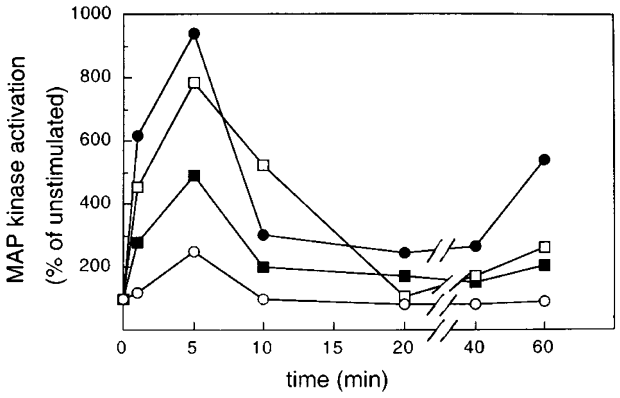

Figure 3 Activation of MAP kinase by stimulation with PDGF-AA, -AB or -BB. PAE/$\alpha\beta$ cells were stimulated with 100 ng/ml of PDGF-AA (□), -AB (●) or -BB (■) and PAE/PDGFRβ cells with PDGF-BB (○) for indicated time periods at 37°C, followed by lysis of cells and immunoprecipitation with an antiserum against MAP kinase. The immunoprecipitates were subjected to an *in vitro* kinase assay in the presence of myelin basic protein (MBP) as an exogenous substrate and then analysed by SDS–PAGE using a 15% polyacrylamide gel. The protein bands corresponding to MBP were quantified using a Fuji BAS 2000 Bioimage Analyzer and plotted relative to control cells, where no ligand was added. The data is a representative one of four experiments

RasGAP binds to the PDGF β-receptor in a homodimeric but not in a heterodimeric receptor complex

RasGAP induces the GTPase activity of Ras and thereby converts active GTP-Ras to inactive GDP-Ras. GAP has been shown to bind to homodimeric β-receptor complexes but not to homodimeric α-receptor complexes (Heidaran *et al*., 1993). It is thus possible

that the binding of GAP to the β-receptor counteracts the activation of Ras that is achieved by the binding of the Grb2-Sos complex to the receptor. We investigated whether a differential interaction between GAP and the PDGF β-receptor could explain the differences in activation of Ras and MAP kinase by the different PDGF isoforms.

GAP immunoprecipitates from PAE/$\alpha\beta$ cells stimulated with different PDGF isoforms were subjected to *in vitro* kinase assay. A ^{32}P-labeled component of the same size as the PDGF β-receptor was observed in GAP immunoprecipitates from cells stimulated with PDGF-BB; a faint band of similar size was also seen after stimulation with PDGF-AB, but not after stimulation with PDGF-AA (Figure 4a). A similar pattern was also seen when stimulated PAE/$\alpha\beta$ cells were subjected to immunoprecipitation with a GAP antiserum followed by blotting with phosphotyrosine antibodies; PDGF-BB induced co-precipitation of a component which most likely is the PDGF β-receptor, PDGF-AB had less effect and PDGF-AA was unable to induce co-immunoprecipitation of the receptor with GAP (Figure 4b). These data are consistent with a more efficient association of GAP with the β-receptor in a homomeric receptor complex than in a heteromeric receptor complex.

To characterize the type of receptors that are activated in response to the different PDGF isoforms, PAE/$\alpha\beta$ cells were serum-starved overnight and stimulated with 100 ng/ml of PDGF-AA, -AB or -BB. Cells expressing the PDGF β-receptor (PAE/PDGFRβ) alone were stimulated with PDGF-BB as a control. As shown in Figure 4c, PDGF-BB gave a stronger tyrosine phosphorylation of the PDGF β-receptor compared to PDGF-AB, which indicates a more efficient activation of homodimeric PDGF β-receptor after stimulation with PDGF-BB compared to PDGF-AB.

Lowered phosphorylation of Tyr771 in the β-receptor in a heteromeric receptor complex

A possible explanation for the lower association of GAP with the β-receptor in complex with the α-receptor, compared to the homomeric β-receptor could be a lowered phosphorylation of Tyr771, which is the known docking site for GAP in the β-receptor (Fantl *et al*., 1992; Kazlauskas *et al*., 1992). To explore this possibility, PAE/$\alpha\beta$ cells were labeled with ^{32}P-orthophosphate and then stimulated with PDGF-BB or PDGF-AB for 60 min. PDGF β-receptors were immunoprecipitated and subjected to tryptic digestion. The tryptic digests were then subjected to immunoprecipitation with a peptide antiserum raised against a synthetic peptide corresponding to the sequence of the tryptic peptide containing Tyr771; these immunoprecipitates were then subjected to Edman degradation. As shown in Figure 5, ^{32}P-radioactivity was released in cycles 1, 9 and 16 of the peptide from PDGF-BB stimulated cells, corresponding to Tyr763, Tyr771, and Tyr778, indicating that all these tyrosine residues are autophosphorylation sites. No appreciable radioactivity was observed in cycle 13 corresponding to Tyr775. In contrast, when the tryptic peptide from PDGF-AB-stimulated cells were subjected to Edman degradation, [^{32}P]radioactivity was released in cycle 1 and very little

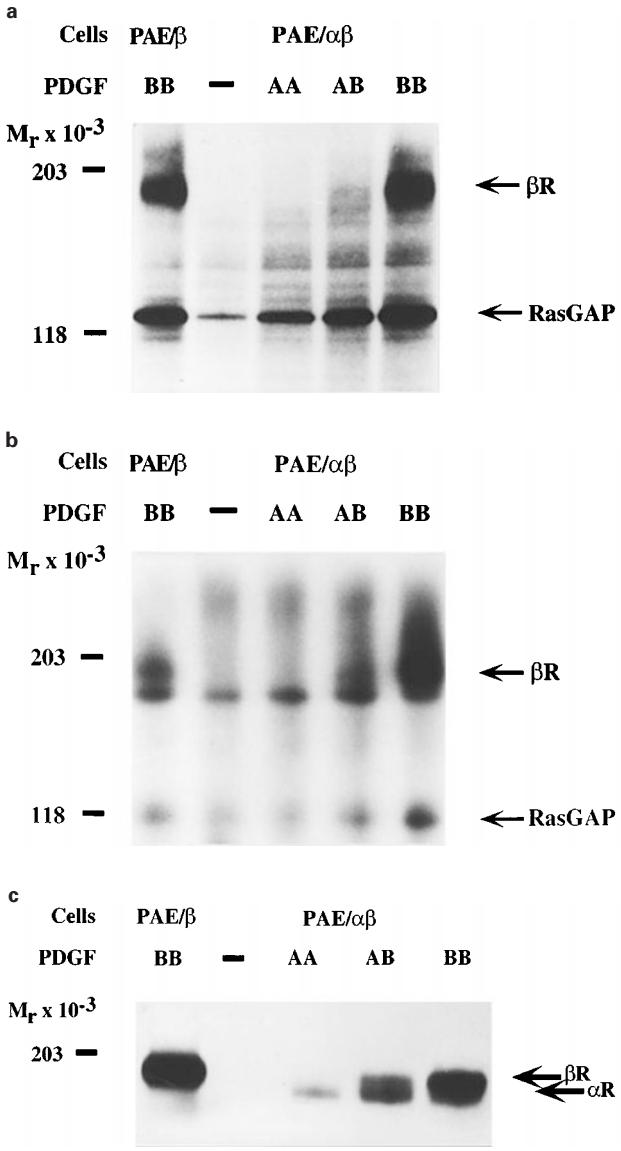

Figure 4 (**a**) Interaction between RasGAP and the stimulated PDGF β-receptor in homo- and heterodimeric complexes. PAE/$\alpha\beta$ cells or PAE/PDGFRβ cells were serum-starved overnight and then stimulated with 100 ng/ml of PDGF-AA, -AB or -BB for 1 h at 4°C followed by another 5 min at 37°C. The cells were lysed and the lysates incubated with antibodies against RasGAP. The immunoprecipitates were then subjected to *in vitro* kinase assay on beads, followed by SDS–PAGE and electrotransfer to a PVDF membrane. The membrane was then treated with 1 M KOH at 55°C for 1 h, and subjected to autoradiography. (**b**) Differential binding of RasGAP to the PDGF-β receptor in homo- and heterodimeric receptor complexes. PAE/$\alpha\beta$ cells or PAE/PDGFRβ cells were serum-starved overnight and then stimulated with 100 ng/ml of PDGF-AA, -AB or -BB for 1 h at 4°C, followed by another 5 min at 37°C. Cells were lysed and incubated with RasGAP antibodies. The immunoprecipitated samples were separated by SDS–PAGE using gradient gels of 5–10% polyacrylamide and then electrotransferred to a PVDF membrane. The membrane was immunoblotted with anti-phosphotyrosine antibodies. The positions of the PDGF β-receptor (βR) and molecular weight markers are indicated to the right and left, respectively of the gels in (**a**) and (**b**) of this figure, and in Figure 6. (**c**) Receptor activation in response to PDGF isoforms. PAE/$\alpha\beta$ cells or PAE cells expressing only the PDGF β-receptor (PAE/PDGFRβ) were serum-starved overnight and then stimulated with 100 ng/ml of PDGF-AA, -AB or -BB for 1 h at 4°C followed by another 5 min at 37°C. The cells were lysed and the lysates incubated with Wheat Germ Lectin Sepharose. The precipitated samples were separated by SDS–PAGE using 5–10% polyacrylamide gels and then electrotransferred to a PVDF membrane. Tyrosine phosphorylation was then detected on the membrane using anti-phosphotyrosine antibodies

in other cycles, indicating that Tyr763 was phosphorylated but not Tyr771, Tyr775 and Tyr778 of the β-receptor in an $\alpha\beta$ receptor heterodimer.

Increased mitogenic response to PDGF-BB in PAE cells expressing wild type α-receptors and a mutant PDGF β-receptor lacking the RasGAP binding site

In order to further evaluate the importance of Tyr771 in the β-receptor in negative regulation of Ras, PAE cell lines were established stably expressing equal levels of wild type PDGF α-receptors and mutant PDGF β-receptors, with Tyr771 replaced with a phenylalanine residue. Three different PAE/$\alpha\beta$Y771F clones, with receptor expression levels comparable with PAE/$\alpha\beta$ cell lines (data not shown), were selected for further studies.

The Y771F mutation in the PDGF β-receptor was analysed in PAE/$\alpha\beta$Y771F cell lines to assess whether this mutation affects the kinase activity of the receptor. PAE/$\alpha\beta$Y771F cells were serum-starved overnight and stimulated with 100 ng/ml PDGF-AA or PDGF-BB for 1 h at 4°C. PAE/$\alpha\beta$ cells and cells expressing only the PDGF α-receptor or the PDGF β-receptor were used as control cell lines. As shown in Figure 6, the Y771F mutation of the PDGF β-receptor did not affect its kinase activity, since PDGF-BB stimulation gives a similar tyrosine phosphorylation of the PDGF β-receptor as in the control cell lines.

The different PDGF isoforms were used to stimulate incorporation of [^{3}H]thymidine in PAE/$\alpha\beta$Y771F cells. As shown in Figure 7, PDGF-BB gave a stronger mitogenic response than PDGF-AA and PDGF-AB. This is to be compared with the mitogenic response in the PAE/$\alpha\beta$ cell line, where PDGF-AB is the most potent isoform in stimulating [^{3}H]thymidine incorporation. Thus, on cells with PDGF β-receptors lacking the RasGAP binding site, PDGF-BB has a more potent mitogenic effect. These results indicate that RasGAP has a role in downregulating the mitogenic response of PDGF.

Increased mitogenic response and MAP kinase activation in a homodimeric PDGF β-receptor complex lacking a RasGAP binding site

In order to evaluate the importance of Tyr771 in homodimeric PDGF β-receptor complexes, PAE cell

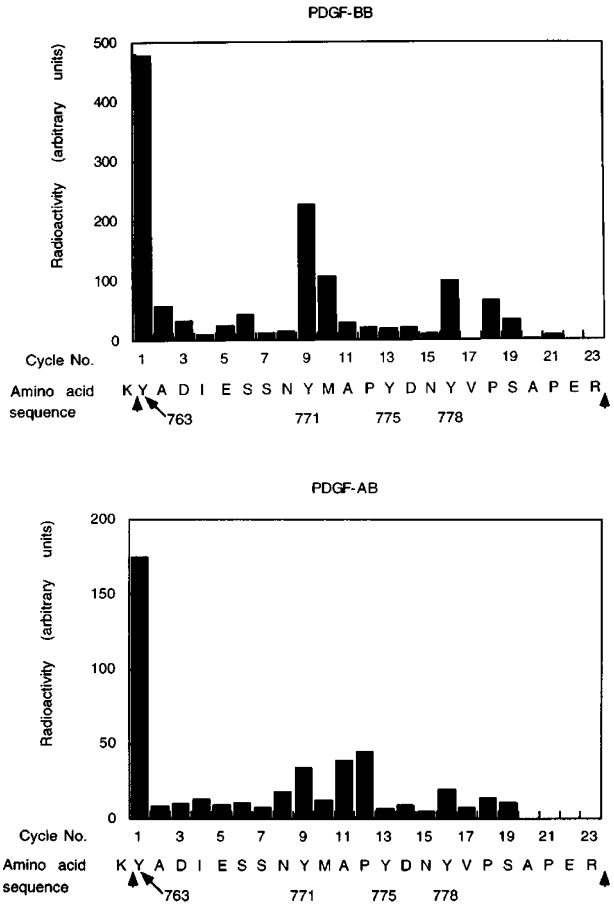

Figure 5 Analysis of phosphorylation of the RasGAP binding site Tyr771 in the PDGF β-receptor in homo- and heterodimeric complexes. PAE/$\alpha\beta$ cells or PAE/PDGFRβ cells were subjected to *in vivo* ^{32}P-orthophosphate labeling and were then stimulated with 100 ng/ml of PDGF-AB or -BB. Cells were lysed and the lysates were incubated with anti-phosphotyrosine antibodies. The immunoprecipitates were eluted from Protein A-Sepharose beads, treated with iodoacetamide to prevent redimerization and immunoprecipitated again with anti-PDGF β-receptor antibodies. The samples were separated by SDS–PAGE and electrotransferred to a nitrocellulose filter. After tryptic digestion of the receptors, the digests were incubated with antibodies directed against the region of the PDGF β-receptor comprising Tyr771 and the eluted immunoprecipitates were then analysed by Edman degradation by the use of a gas phase sequencer. The release of radioactivity in each cycle is indicated as arbitrary units. The deduced sequence in the PDGF β-receptor is shown with the tryptic cleavage sites indicated by arrows. The upper figure is from PDGF-BB stimulated cells and the lower figure is from PDGF-AB stimulated cells. The sequence of the immunoprecipitated tryptic sequence is shown. Tryptic cleavage sites are indicated by arrow heads

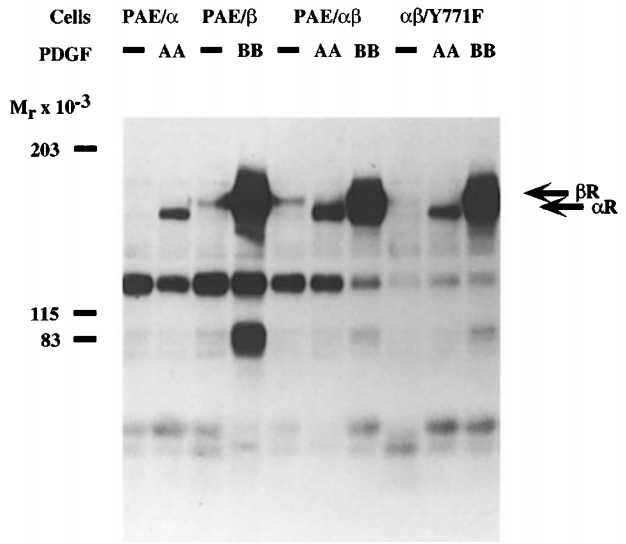

Figure 6 Ligand-stimulated kinase activity of Y771F mutated PDGF β-receptor. PAE/$\alpha\beta$Y771F cells, PAE/$\alpha\beta$ cells or PAE cells expressing either wild type PDGF α-receptor (PAE/PDGFRα) or PDGF β-receptor (PAE/PDGFRβ) alone were serum-starved overnight and then stimulated or not with 100 ng/ml PDGF-AA or PDGF-BB for 1 h at 4°C. The cells were lysed and the lysates incubated with Wheat Germ Lectin Sepharose. The precipitated samples were separated by SDS–PAGE using 5–10% polyacrylamide gels and then electrotransferred to a PVDF membrane. The membrane was immunoblotted with anti-phosphotyrosine antibodies

lines were established expressing mutant PDGF β-receptors with a tyrosine to phenylalanine substitution at position 771. Two different PAE/βY771F cell clones, with receptor expression comparable with those of PAE/$\alpha\beta$Y771F cell lines, were selected for further studies.

As shown in Figure 8a, the Y771F mutant PDGF β-receptor mediated a significantly stronger mitogenic response compared to the wild type receptor as determined by a [^{3}H]thymidine incorporation assay. This result suggests that a phosphorylated Tyr771 allowing binding of RasGAP in the negative regulation of mitogenicity, and is consistent with the finding that PDGF-BB gave a strong mitogenic response in PAE/$\alpha\beta$Y771 cells (Figure 7).

In order to investigate the importance of the Y771F mutation for MAP kinase activation, PAE/βY771F cells and PAE/PDGFRβ cells were stimulated with PDGF-BB for different time periods. MAP kinase activity was then measured using myelin basic protein as a substrate. As shown in Figure 8b, the Y771F mutant PDGF β-receptor mediated a significantly higher activation of MAP kinase after 5 min of PDGF-BB stimulation compared to the wild type receptor. This data indicates the importance of RasGAP in negative regulation of the Ras/MAP kinase pathway and can help to explain the strong effect of PDGF-AB on mitogenicity and Ras/MAP kinase activation observed in PAE/$\alpha\beta$ cells.

Discussion

PDGF-stimulation leads to an activation of Ras and the MAP kinase cascade, through the binding of the Grb2/Sos1 complex to the autophosphorylated receptor or to components associated with the receptor. The stimulatory signal is, however, modulated by an inhibitory signal exerted by the binding of GAP to the activated receptor, which converts active Ras·GTP to inactive Ras·GDP. The notion that GAP exerts such a negative control function is supported by the finding that PDGF-induced Ras activation is increased in cells from mice with the GAP gene inactivated (van der Geer *et al*., 1997). Thus the net activation of Ras is determined by the balance between stimulatory and inhibitory signals.

We show in this report that Tyr771 is phosphorylated in a homodimer of PDGF β-receptors, but not in an $\alpha\beta$ receptor heterodimer. The consequence is that GAP can bind to a homodimeric but not to a heterodimeric receptor complex. The inability of the heterodimeric receptor complex to bind GAP correlates with an increased ability to activate Ras and MAP kinase, and

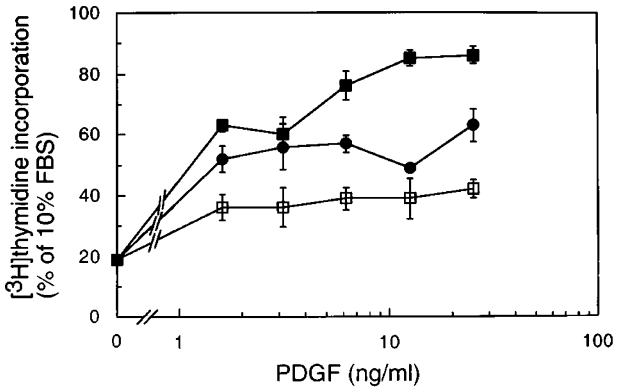

Figure 7 Effect of PDGF isoforms on mitogenicity of PAE/$\alpha\beta$Y771F cells. After 24 h of starvation in 1 mg/ml BSA, PAE/$\alpha\beta$Y771F cells were incubated with [^{3}H]thymidine at the indicated concentrations of PDGF-AA (□), PDGF-AB (●) or PDGF-BB (■) for 24 h at 37°C. Incubation with 10% FBS was used as a positive control. After incubation, trichloroacetic acid-precipitated [^{3}H]thymidine radioactivity was measured. Data represent mean values of triplicates with indicated standard deviation and are expressed as percentage of the value obtained for stimulation with 10% FBS. The experiment was repeated four times; a representative result is shown

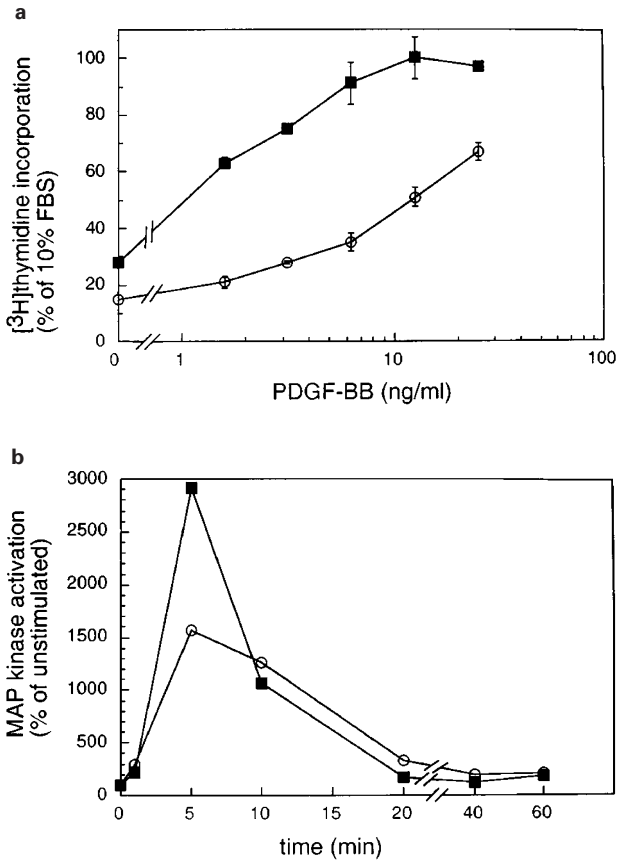

Figure 8 Efficient mitogenic response in PAE/βY771F cells induced by PDGF-BB. After 24 h of starvation in 1 mg/ml BSA, PAE/βY771F (■) or PAE/PDGFRβ (○) cells were incubated with [^{3}H]thymidine at the indicated concentrations of PDGF-BB for 24 h at 37°C. Incubation with 10% FBS was used as a positive control. After incubation, trichloroacetic acid-precipitated radioactivity was measured. Data represent mean values of triplicates with indicated standard deviation and are expressed as percentage of the value obtained for stimulation with 10% FBS. Shown is a representative result; the experiment was repeated four times. (**b**) Efficient MAP kinase activation in PAE/βY771F cells upon PDGF-BB stimulation. PAE/PDGFRβ (○) cells and PAE/βY771F (■) cells were stimulated with 100 ng/ml of PDGF-BB for different time periods at 37°C. The cells were lysed and the lysates incubated with an antiserum against MAP kinase. The immunoprecipitates were subjected to an *in vitro* kinase assay in the presence of myelin basic protein (MBP) as an exogenous substrate and then analysed by SDS–PAGE using a 15% polyacrylamide gel. The radioactive protein bands corresponding to MBP were quantified using a Fuji BAS 2000 Bioimage Analyzer and plotted as percentage of control cells, where no ligand was added. The data is a representative one of four experiments

to an increased mitogenic signaling. The notion that GAP binding to receptor complexes exerts a negative control mechanism is further supported by the finding that PDGF-BB gives a more potent mitogenic effect than PDGF-AB in cells expressing wild type α-receptors together with a Y771F mutant β-receptor unable to bind GAP, whereas the reverse is true in cells with wild type α-receptors and wild type β-receptors.

The reason why Tyr771 is phosphorylated in a homodimeric ββ receptor complex but not in the heterodimeric αβ receptor complex is not known. It is possible that the substrate specificity of the α- and β-receptor kinases differ, so that Tyr771 can be recognized as a phosphoacceptor site by the β-receptor but not by the α-receptor. However, when a chimera of the PDGF α-receptor with the PDGF β-receptor kinase insert (Arvidsson *et al*., 1992) was assayed for phosphorylation of the tyrosine residue corresponding to Tyr771 in the PDGF β-receptor, the degree of phosphorylation was as in the wild type PDGF β-receptor (data not shown). This suggests that the lack of phosphorylation of Tyr771 in the PDGF β-receptor is not due to the specificity of the PDGF α-receptor kinase. Alternatively, the α-receptor, but not the β-receptor, recruits and activates a phosphatase which efficiently dephosphorylates Tyr771 in the β-receptor. A third possibility is that a hitherto unknown tyrosine kinase associates with the PDGF β-receptor, but not with the PDGF α-receptor, and phosphorylates Tyr771.

Our observations provide an illustration that heterodimeric complexes of tyrosine kinase receptors can have different properties compared to the corresponding homodimeric complexes, and also provide a molecular mechanism herefore, i.e. differences in autophosphorylation. Analogously, functional differences between various heterodimeric and homodimeric complexes of members of the EGF receptor family have also been reported (Graus-Porta *et al*., 1997; Pinkas-Kramarski *et al*., 1996). It is possible that the ability to form heterodimeric complexes within structurally related subfamilies is a general property of tyrosine kinase receptors. Whether homo- or heteromeric complexes will be formed depend on the receptor binding specificity of the stimulating ligand as well as on the level of expression of different receptors on the target cell. This provides a mechanism whereby the spectrum of responses can be enlarged using a limited number of receptors.

Materials and methods

Plasmids, cells and cell culture

A cDNA encoding the full-length PDGF α-receptor (Claesson-Welsh *et al*., 1989) was inserted into the eukaryotic expression vector pcDNA3 (Invitrogen). Another eukaryotic expression vector, pBabe, containing a puromycin resistance gene was used for cotransfection of cells (Morgenstern and Land, 1990). These two constructs were cotransfected into porcine aortic endothelial (PAE) cells expressing the wild type PDGF β-receptor, using electroporation. Stable transfectants were obtained by selection with puromycin (Calbiochem) at a concentration of 0.5 μg/ml. The PAE cells were maintained as previously described (Mori *et al*., 1991). For starvation, subconfluent cell cultures were incubated for 16 h in Ham's F-12 medium containing 0.3% fetal bovine serum (FBS, Gibco).

Antibodies and ligands

The peptide antisera PDGFR-3 and PDGFR-7, directed against the carboxyterminal tails of PDGF β- and α-receptors, respectively, have been described previously (Eriksson *et al*., 1992; Mori *et al*., 1993). The monoclonal anti-phosphotyrosine antibody PY20 was purchased from Transduction Laboratories. Antiserum against p120RasGAP was from Santa Cruz Biotechnology Inc. Peroxidase-conjugated sheep anti-mouse was from Amersham Corp. Rabbit anti-mouse Ig antibody and rabbit anti-rat Ig antibody were from Dakopatts. An antiserum recognizing amino acid residues 771–789 in the human PDGF β-receptor was a kind gift of Dr Thomas O Daniel, Vanderbilt University (Nashville, TN, USA) (Kumjian *et al*., 1989). A rabbit antiserum reacting with Erk2 was raised against the carboxyterminal sequence EETARFQPGYRS (Leevers and Marshall, 1992). PDGF-AA, PDGF-BB and PDGF-AB were kind gifts from Amgen.

Immunoprecipitation and Western blotting

Cells were starved overnight in Ham's F-12 medium containing 0.3% FBS, preincubated with 100 μM Na_3VO_4 and then treated with 100 ng/ml PDGF for 1 h at 4°C followed by 5 min at 37°C. Cells were rinsed twice with ice-cold phosphate-buffered saline (PBS) and lysed in 1% Triton X-100, 10% glycerol, 100 mM NaCl, 20 mM HEPES, pH 7.4, 5 mM EDTA, 1% Trasylol, 1 mM phenylmethylsulfonyl fluoride (PMSF), 200 μM Na_3VO_4, 10 μg/ml leupeptin (Boehringer Mannheim). Lysates were incubated at 4°C with 3.5 μg of α-GAP antibody (Santa Cruz) per sample for 2 h, followed by incubation with Protein A-Sepharose 6MB (Pharmacia Biotech) for 30 min at 4°C. Lysates were also incubated with 100 μl 1 : 1 slurry of Wheat germ Lectin Sepharose 6 MB (Pharmacia Biotech) for 1.5 h end-over-end at 4°C to enrich a glycoprotein fraction. The immunoprecipitates were washed four times with lysis buffer and once with 20 mM HEPES, pH 7.4, and then subjected to SDS-polyacrylamide electrophoresis.

For Western blotting, the proteins were electrotransferred from the polyacrylamide gel to a polyvinylidene difluoride (PVDF) membrane, which were then blocked with PBS containing 0.2% Tween 20. The blots were then incubated with anti-phosphotyrosine antibody PY20 (1:2000 dilution), washed and incubated with horseradish peroxidase-conjugated sheep anti-mouse immunoglobulins (1:5000 dilution). After washing, the proteins were visualized using the ECL Western blotting detection system (Amersham).

In vitro *kinase assay*

The *in vitro* kinase assays were performed essentially according to Hansen *et al*. (1996). After treatment with PDGF, the cells were lysed in Triton X-100 lysis buffer supplemented with 1 mM dithiothreitol (DTT) and the lysates were then incubated with respective antibody. After Protein A-Sepharose incubation, the samples were subjected to kinase assay and analysed by SDS–PAGE. The proteins were electrotransferred to a PVDF membrane; the blots were then treated with 1 M KOH at 55°C for 1 h according to Kamps and Sefton (1989) to remove background due to serine phosphorylation, and subjected to autoradiography.

In vivo ^{32}*P-orthophosphate labeling*

Labeling was performed according to Rönnstrand *et al*. (1992), with some modifications. Briefly, subconfluent cells were washed three times with phosphate-free Ham's F-12 medium supplemented with 0.1% dialyzed fetal calf serum and 20 mM HEPES. Cells were then labeled in phosphate-

free medium containing 3 mCi/ml ^{32}P-orthophosphate for 3 h at 37°C. Cells were stimulated with 100 ng/ml of PDGF for 1 h at 4°C in the same medium, washed in ice-cold PBS and then lysed in 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 20 mM HEPES pH 7.4, 5 mM EDTA, 30 mM $Na_4P_2O_7$, 1% Trasylol, 1 mM PMSF, 200 μM Na_3VO_4, 10 μg/ml leupeptin. Immunoprecipitation was performed using anti-phosphotyrosine antibody PY20 in the presence of 100 μg of RNaseA and 50 μg of DNaseI (Boehringer Mannheim). After incubation with Protein A-Sepharose, the samples were washed with lysis buffer, heated at 95°C for 2 min in Tris buffered saline (TBS) containing 1% SDS and 10 mM DTT and then incubated with 50 mM of iodoacetamide for 15 min at room temperature. Supernatants were diluted ten times with lysis buffer and subjected to immunoprecipitation using antisera against PDGF α-receptor (PDGF-R7) or β-receptor (PDGF-R3). The samples were separated by SDS–PAGE and then electrotransferred to a nitrocellulose membrane (Hybond C-Extra, Amersham). The bands corresponding to the phosphorylated PDGF β-receptor were cut out and subjected to tryptic cleavage.

In situ *trypsin digestion and immunoprecipitation after tryptic cleavage*

Phosphorylated proteins were separated by SDS–PAGE and electrotransferred to nitrocellulose membrane (Hybond C-extra, Amersham). Tryptic digestion was performed essentially according to Mori *et al*. (1993). Immunoprecipitation of tryptic peptides, with antibodies covalently coupled to Protein A-Sepharose, was performed according to Hansen *et al*. (1996). After elution with 1% diethylamine, the peptides were covalently coupled to Sequelon AA membranes according to the manufacturer's description and subjected to Edman degradation in an Applied Biosystems Gas Phase Sequenator (Model 477A), as described by Blume-Jensen *et al*. (1995).

Mitogenic assay

PAE cells expressing equal levels of PDGF α- and β-receptors were seeded in 12-well plates and grown for 24 h. While cells were still subconfluent, the medium was changed to starvation medium (Ham's F-12, 0.3% FBS). After 24 h of starvation, 0.2 μCi [^{3}H]thymidine/ml (Amersham) and PDGF (0–25 ng/ml) or 10% FBS were added and incubation continued for 20 h. Macromolecules were then precipitated using 5% trichloracetic acid (TCA), samples were washed three times in ice-cold H_2O and dissolved in 1 M NaOH, followed by neutralization with 2 M HCl. [^{3}H]radioactivity was measured in a liquid scintillation counter.

MAP kinase assay

Cells were starved overnight in 0.3% FCS and then stimulated with 100 ng/ml of PDGF for indicated time periods at 37°C. Cells were lysed in 1% Triton X-100, 0.5% sodium deoxycholate, 20 mM HEPES, pH 8.0, 10 μg/ml leupeptin (Boehringer Mannheim), 1% aprotinin, 1 mM PMSF, 20 mM $Na_4P_2O_7$, 200 μM Na_3VO_4, 10 mM EGTA, 5 mM $MgCl_2$, 1 mM DTT and lysates incubated with a rabbit antiserum against MAP kinase for 2 h at 4°C, as described above. The immunoprecipitated samples were washed three times with the lysis buffer, once with 20 mM HEPES, pH 8.0, and twice with a kinase buffer (20 mM HEPES, pH 8.0, 20 mM $MgCl_2$, 2 mM $MnCl_2$, 1 mM DTT). The samples were subjected to an *in vitro* kinase assay for 15 min at 30°C in 40 μl kinase buffer supplemented with 5 μCi [γ-^{32}P]ATP and 10 μg myelin basic protein (MBP; GIBCO BRL). Reactions were stopped by addition of SDS-sample buffer, samples were boiled for 5 min and separated on a 15% SDS-polyacrylamide gel. Quantification of radioactivity incorporation into protein bands corresponding to MBP was done using a Bioimage analyzer BAS 2000 (Fuji).

Detection of guanine nucleotides bound to Ras

Ras bound guanine nucleotides was measured at different time points after stimulation with either PDGF-AA, PDGF-BB or PDGF-AB, in cells starved overnight in Ham's F-12, 0.3% FBS, essentially according to Burgering *et al*. (1991). Guanine nucleotides were quantified using a Fuji BAS 2000 Bioimage Analyzer.

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