Loop III region of platelet-derived growth factor (PDGF) B-chain mediates binding to PDGF receptors and heparin

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Site-directed mutagenesis of the platelet-derived growth factor (PDGF) B-chain was conducted to determine the importance of cationic amino acid residues (Arg¹⁶⁰-Lys¹⁶¹-Lys¹⁶²; RKK) located within the loop III region in mediating the biological and cell-association properties of the molecule. Binding to both PDGF α - and β -receptors was inhibited by the conversion of all three cationic residues into anionic glutamates (RKK $\rightarrow$ EEE), whereas an RKK $\rightarrow$ SSS mutant also exhibited a modest loss in affinity for β -receptors. Replacements with serine at either Arg¹⁶⁰ (RKK $\rightarrow$ SKK) or at all three positions (RKK $\rightarrow$ SSS) had little effect on binding to α -receptors. Replacements with either glutamic or serine residues at any of the three positions also resulted in significant inhibition of heparin-binding activity. Furthermore, the RKK $\rightarrow$ EEE mutant exhibited decreased association with

the cell surface and accumulated in the culture medium as 29–32 kDa forms. Stable transfection of U87 astrocytoma cells with RKK $\rightarrow$ EEE mutants of either the A-chain or the B-chain inhibited malignant growth in athymic nude mice. Despite altered receptor-binding activities, each of the loop III mutants retained full mitogenic activity when applied to cultured Swiss 3T3 cells. CD spectrophotometric analysis of the RKK $\rightarrow$ EEE mutant revealed a secondary structure indistinguishable from the wild type, with a high degree of β -sheet structure and random coil content (50 % and 43 % respectively). These findings indicate an important role of the Arg¹⁶⁰-Lys¹⁶¹-Lys¹⁶² sequence in mediating the biological and cell-associative activities of the PDGF-BB homodimer, and reveal that the mitogenic activity of PDGF-BB is insufficient to mediate its full oncogenic properties.

INTRODUCTION

Platelet-derived growth factor (PDGF) is a potent mitogen and chemoattractant that has critical roles in embryogenesis, cell differentiation and wound repair. PDGF is a family of three disulphide-linked glycoprotein dimers of molecular masses 29–32 kDa that arise from stochastic assembly of the homologous subunits, A-chain and B-chain, yielding the heterodimer PDGF-AB and homodimers PDGF-AA and PDGF-BB (reviewed in [1]). The A-chain is expressed as two different forms ('long' and 'short') as determined by alternative splicing, the long form containing an extended C-terminus of basic amino acids that mediates association with the extracellular matrix and is encoded by exon 6 [2]. The B-chain also contains a cationic C-terminus that has been implicated in the strong association of PDGF-BB with the cell surface [3]. PDGF action is mediated by two PDGF receptor isotypes, α and β , which on ligand binding associate non-covalently to form homo- and hetero-dimers [4,5]. PDGF α -receptor has a high affinity for both A- and B-chains, whereas the β -receptor recognizes only the B-chain [6].

X-ray crystallographic analysis of recombinant human PDGF-BB has revealed an antiparallel arrangement of the disulphide-linked B-chain monomers [7]. Each B-chain monomer is characterized by a so-called 'cysteine knot' structure, consisting of three intrachain disulphide bonds and two antiparallel β -strands, interconnected by three loop segments (I–III). We have demonstrated that the cationic residues Arg¹⁵⁹, Lys¹⁶⁰ and Lys¹⁶¹

of the A-chain, which correspond to the loop III region of the B-chain, are required for high-affinity receptor binding and mitogenic activity [8]. Furthermore, a synthetic peptide containing loop III residues 157–163 of the B-chain exhibited PDGF-antagonizing activity [9], whereas a mutation at residue Lys¹⁶¹ of the B-chain was recently shown to interfere with high-affinity binding to the α -receptor [10].

To assess the functional importance of the cationic loop III amino acids of the B-chain in greater detail, three mutations were constructed and analysed for their abilities to inhibit receptor binding and mitogenic activity. The mutant proteins were also assessed for binding to the glycosaminoglycan heparin and their ability to inhibit the transformed growth of cultured human astrocytoma cells via a dominant-negative mechanism.

MATERIALS AND METHODS

Expression of recombinant proteins in Chinese hamster ovary (CHO) cells

The development of CHO cells expressing human PDGF B-chain [designated WT-RKK, for wild-type loop III residues Arg¹⁶⁰-Lys¹⁶¹-Lys¹⁶² (RKK)] has been described [11]. Overlap extension PCR [12] was used to introduce a mutation converting the three cationic amino acids of loop III into glutamic residues (mutant RKK $\rightarrow$ EEE). Mutant B-chain cDNA species were

Abbreviations used: CHO, Chinese hamster ovary; ED₅₀, half-maximal effective dose; HS-PG, heparan sulphate proteoglycan; PDGF, platelet-derived growth factor; RKK, Arg-Lys-Lys; WT-RKK, wild-type PDGF-BB; FGF, fibroblast growth factor.

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expressed in DUX B11 cells, a dihydrofolate reductase-deficient variant of CHO cells, under control of the Rous sarcoma virus promoter. Fidelity of all PCR syntheses was verified by DNA sequencing. Methods for stable transfection, methotrexate-induced gene amplification and screening of culture supernatants for production of PDGF B-chain have been described [11].

Partial purification of WT-RKK and mutant RKK → EEE from CHO cells

PDGF-BB-containing culture supernatants were collected over a 96 h period in serum-free Dulbecco's modified Eagle's medium as described [8]. Proteins were partly purified by heparin-agarose (Sigma) chromatography (2.5 cm × 5 cm column), concentrated by ultrafiltration (Centriprep and Centricon 20; Amicon), and stored at -80 °C in the presence of 10 % (v/v) glycerol.

Preparation of wild-type and mutant PDGF-BB from *Pichia pastoris*

To obtain sufficient quantities of wild-type and mutant forms of PDGF B-chain for purification and analysis, cDNA species were constructed by PCR and inserted into the expression vector pPIC9 for expression in the yeast *P. pastoris* (Invitrogen). Truncated cDNA species corresponding to the mature human B-chain (Ser⁸² to Ala²⁴¹) were inserted in reading frame with the the *Saccharomyces cerevisiae* α factor prepropeptide α factor sequence. cDNA species encoding mutants RKK → SKK and RKK → SSS were obtained by using 'megaprimer' PCR [13]. *P. pastoris* cells were transformed by formation of spheroplasts as described by the supplier.

For crude PDGF-BB preparations from *P. pastoris*, cultures were grown for 48 h in 1 litre of buffered glycerol-complex medium [BMGY: 1 % (w/v) yeast extract/2 % (w/v) peptone/100 mM potassium phosphate (pH 6.0)/1.34 % (w/v) yeast nitrogen base/0.4 µg/ml biotin/1 % (v/v) glycerol] at 30 °C with vigorous shaking (250 rev./min). Recombinant protein expression was induced by suspension in 100 ml of BMMY [composition as for BMGY, with a substitution of 0.5 % (v/v) methanol for glycerol as carbon source] supplemented with casamino acids, followed by growth for 16 h at 30 °C in 1 litre baffled flasks. Culture supernatants were obtained by centrifugation at 6088 g for 15 min at 4 °C, and PDGF-BB was precipitated by the addition of crystalline (NH₄)₂SO₄ (60 % satn.) and centrifugation (20463 g, 60 min). Pellets were resuspended in 50 mM NaCl/40 mM Hepes (pH 7.4), and residual (NH₄)₂SO₄ was removed by ultrafiltration. Proteins were stored at -80 °C in 50 mM NaCl/40 mM Hepes (pH 7.4) containing 10 % (v/v) glycerol (PDGF freezing buffer).

Radioreceptor and [³H]thymidine incorporation assays for PDGF-BB

Radioreceptor assays were conducted in A204 and rat mesangial cells (RMC, R52 line) as described [8,14] with 20000 c.p.m. of [¹²⁵I]-PDGF-BB (Amersham; 1200 Ci/mmol) as radioligand and unlabelled recombinant PDGF-BB proteins as competitor. Mitogenic activities of recombinant PDGF-BB proteins were determined by measuring [³H]thymidine incorporation into cells as described [8].

PDGF receptor autophosphorylation *in vitro*

The assay *in vitro* of PDGF receptor autophosphorylation was conducted essentially as described [15]. A204 and R52 cells were grown to near-confluence on 100 mm plastic dishes and main-

tained in low-mitogenicity medium for 24 h. Cells were prechilled at 4 °C for 60 min and then incubated with PDGF-BB preparations for 10 min at 4 °C. Cell lysates were incubated with either anti-(PDGF receptor α or anti-PDGF receptor β) IgG (Santa Cruz Biotechnologies) for 60 min at 4 °C, centrifuged for 10 min and incubated in fresh tubes containing Protein A-agarose (Sigma) for 60 min at 4 °C. Kinase assays were performed in 25 µl of kinase buffer [20 mM Tris/HCl (pH 7.5)/1 mM dithiothreitol/10 mM MnCl₂] and 5 µCi of [γ -³²P]ATP (3000 Ci/mmol) for 10 min at 30 °C. Immunoprecipitates were analysed by SDS/PAGE [6 % (w/v) gel].

Analysis of heparin-binding activity by batch assay *in vitro*

Mixtures containing 300 µl of a 50:50 slurry of heparin-agarose in 200 mM NaCl/40 mM Hepes (pH 7.4) were incubated with 3 µg of PDGF-BB proteins for 15 min at 23 °C. Supernatants were obtained by microcentrifugation and collected with a 23-gauge needle. Pellets were washed with 300 µl of buffer, which was combined with the first supernatant ('unbound' PDGF). Pellets were then washed twice with 150 µl of buffer containing 1 M NaCl, and supernatants were pooled (high-salt wash fraction, or 'bound' PDGF). The PDGF-BB contents of bound and unbound fractions were determined by ELISA.

Metabolic labelling of CHO cells with [³⁵S]cysteine and SDS/PAGE analysis

WT-RKK, 33 nM methotrexate-selected, and RKK → EEE cells (5 × 10⁵ per 35 mm dish) were pulse-labelled for 30 min with [³⁵S]cysteine (Amersham) (167 µCi in 0.5 ml; 401 Ci/mmol) and chased with a 100-fold molar excess of unlabelled cysteine as described [16]. One of two sets of cells was incubated for 10 min in the presence of 1 mM suramin (Sigma) to release cell-surface-bound PDGF-BB. Immunoprecipitations, SDS/PAGE and autoradiography were performed as described [11].

Purification of *Pichia*-expressed PDGF-BB proteins to apparent homogeneity

To obtain WT-RKK for purification, the procedure described above was scaled up 4-fold. The protein was precipitated from supernatants with (NH₄)₂SO₄ (60 % satn.), resuspended in 40 mM Hepes, pH 7.4, and applied to a column of phenyl-Sepharose (2.5 cm × 7.5 cm). PDGF-BB was eluted with 100 ml of a 50 % solution of ethylene glycol in 40 mM Hepes/150 mM NaCl/20 mM Tris (pH 7.4). The protein was then concentrated, exchanged into 40 mM Hepes, pH 7.4, by ultrafiltration (Amicon), and applied to a column of Q Sepharose (2.5 cm × 4 cm; Pharmacia Fast Flow). The protein was eluted with a linear 200 ml NaCl gradient (0.05–1.5 M), concentrated and exchanged into 40 mM Hepes, pH 7.4, by ultrafiltration. Protein was then applied to a 1 cm × 7 cm column of heparin-agarose (Sigma), equilibrated with 100 mM NaCl/40 mM Hepes (pH 7.4) and eluted with 600 mM NaCl. PDGF-BB was concentrated, applied to a 1 cm × 8 cm column of Sephadex G-25 (Sigma) equilibrated with 0.5 M urea/10 mM glycine pH 3.0, and further purified by Superose 12 FPLC (Pharmacia; 10 cm × 30 cm), as described [17].

The *Pichia* clone expressing RKK → EEE was grown on a large scale (14 litres) under conditions optimized for high requirements of methanol and O₂, with a New Brunswick Microferm Fermenter. Cleared culture supernatant (5 litres) was obtained by Sharples centrifugation, yielding approx. 83 mg of PDGF-BB. After precipitation of protein from the culture

supernatant with 80 %-satd. $(\text{NH}_4)_2\text{SO}_4$, one-sixth of the precipitate was subjected to phenyl-Sepharose chromatography (2.5 cm $\times$ 18 cm column). The protein was eluted in 300 ml of 50 % (v/v) ethylene glycol, concentrated and exchanged into 50 mM NaCl/10 mM Na_2HPO_4 (pH 7.4) by ultrafiltration. The protein was applied to a 5 cm $\times$ 5 cm column of Mono-Q at a flow rate of 1 ml/min and eluted with a linear 200 ml gradient (0.15–1 M NaCl/10 mM Na_2HPO_4). It was further purified by Superose 12 FPLC (10 cm $\times$ 30 cm) in a buffer of 150 mM NaCl/10 mM Na_2HPO_4 (pH 7.4) (flow rate 0.4 ml/min). PDGF-containing fractions were pooled, concentrated and stored in PDGF freezing buffer at -80°C until further use. Protein concentrations were determined by amino acid compositional analysis.

CD spectrophotometry

Spectra of purified RKK $\rightarrow$ EEE were recorded in the wavelength range 190–260 nm with a Jasco 600 CD spectrometer and a 0.01 cm-path-length circular demountable cell. The protein was analysed at a concentration of 0.20 mg/ml in 10 mM acetic acid, pH 3.2. The mean residue molecular mass of RKK $\rightarrow$ EEE was estimated as 235 Da, using an estimate of molecular mass for the PDGF-BB dimer of 26 kDa. Estimates of secondary structure were derived by using software supplied by Jasco, which is based on the method of Yang et al. [18].

Stable transfection of human glioblastoma (U87) cells with PDGF expression vectors and tumour growth in athymic nude mice

Transfections and stable expression in the human glioblastoma cell line U87 were performed with a liposomal reagent (Lipofectin; Gibco/BRL), as described [19]. Cells were cultured in 162 cm² flasks in complete Dulbecco's modified Eagle's medium. On the day of injection, cells were removed by trypsin treatment, rinsed twice with Hanks balanced salt solution (without Phenol Red; Gibco/BRL), and resuspended in 300 μl of Hanks balanced salt solution. Cells (10^7) from exponentially growing cultures of stable U87 transfectants were injected subcutaneously between the scapulae of 9-week-old athymic nude mice (Harlan Sprague-Dawley, *nu/nu*) using a 1 ml tuberculin syringe equipped with a 23-gauge needle. Two-dimensional measurements of tumours were obtained with vernier calipers.

RESULTS

Mutagenesis of the Arg¹⁶⁰-Lys¹⁶¹-Lys¹⁶² sequence of the PDGF B-chain disrupts the ability to bind PDGF α - and β -receptors and induce receptor autophosphorylation

Cationic amino acid residues located within the loop III region of the B-chain (Arg¹⁶⁰-Lys¹⁶¹-Lys¹⁶²; RKK) were replaced with anionic glutamatic residues or polar, uncharged serine residues to analyse the role of this region in B-chain function. In addition to WT-RKK, three mutant proteins were expressed in the methylotrophic yeast *P. pastoris* for functional and structural analyses. Two of the mutations consisted of replacements of the Arg¹⁵⁹-Lys¹⁶⁰-Lys¹⁶¹ sequence at each residue with either glutamate (Arg¹⁵⁹-Lys¹⁶⁰-Lys¹⁶¹ to Glu¹⁵⁹-Glu¹⁶⁰-Glu¹⁶¹; RKK $\rightarrow$ EEE) or serine (RKK $\rightarrow$ SSS), whereas the third contained a single Arg¹⁵⁹ to Ser¹⁵⁹ substitution (RKK $\rightarrow$ SKK). Serine was selected owing to its non-charged but hydrophilic character, which was predicted to minimize structural disruption of the external loop III domain. To permit independent assessment of the role of the RKK motif for binding to PDGF α - and β -

Figure 1 Site-directed mutagenesis of cationic residues located within the loop III region of PDGF B-chain disrupts binding to PDGF α - and β -receptors

Wild-type (WT-RKK, $\square$) and mutant [RKK $\rightarrow$ EEE ($\circ$), RKK $\rightarrow$ SSS ($\bullet$) and RKK $\rightarrow$ SKK ($\blacktriangledown$)] PDGF-BBs were expressed in the methylotrophic yeast *P. pastoris* and concentrated from culture supernatants by $(\text{NH}_4)_2\text{SO}_4$ precipitation and ultrafiltration. The proteins were analysed for their relative abilities to compete for binding of ^{125}I -PDGF-BB to α -receptors [A204 cells (**A**)] and β -receptors [rat mesangial R52 cells (**B**)]. Results are expressed as percentages of maximum specific binding of radioligand to cells and are representative of several experiments performed with at least two different PDGF-BB preparations. Concentrations of PDGF-BB proteins were determined by ELISA and are shown relative to an RKK $\rightarrow$ EEE preparation for which protein concentration was determined directly by amino acid compositional analysis.

receptors, the effects of these mutations were evaluated in both A204 cells, which express exclusively the α isotype [20], and R52 cells, which express the β [11].

Compared with the WT-RKK protein, the RKK $\rightarrow$ EEE mutant was markedly impaired (by a factor of 400) in its ability to compete with ^{125}I -PDGF-BB for binding to both A204 (Figure 1A and Table 1) and R52 cells (Figure 1B and Table 1), indicating that one or more residues in the RKK sequence are involved in the binding of PDGF-BB to both α - and β -receptors. In addition the RKK $\rightarrow$ SSS mutant exhibited a significant decrease in slope and a slight decrease in affinity (3-fold) when analysed in R52 cells, although no effect of this mutation was seen on binding to A204 cells. This contrast reveals a subtle difference in the nature of the interaction of the RKK sequence with the two different receptor subclasses. Binding of the RKK $\rightarrow$ SKK mutant to PDGF receptors was unaffected in either

Table 1 Biological activity of PDGF-BB mutants

IC₅₀ and ED₅₀ results were derived from radioreceptor and mitogenesis assay data shown in Figures 1 and 3 respectively. IC₅₀ and ED₅₀ values were calculated by using the four-parameter logistic equation described by the ALLFIT algorithm [41].

Protein	IC ₅₀ (ng/ml)		ED ₅₀ (ng/ml)
	α	β	
WT-RKK	9.2	4.0	52
RKK → SKK	2.3	2.2	28
RKK → SSS	6.5	13.8	12
RKK → EEE	4040	1240	52

Figure 2 Replacement of the Arg¹⁶⁰-Lys¹⁶¹-Lys¹⁶² sequence of PDGF-BB with glutamate residues decreases the ability to induce α - and β -receptor autophosphorylation

The relative abilities of PDGF to induce α - and β -receptor autophosphorylation were determined by a kinase assay *in vitro*, as described in the Materials and methods section. Radiolabelled proteins were resolved by SDS/PAGE [6% (w/v) gel] under reducing conditions. Indicated at the left are the relative mobilities of pre-stained myosin (205 kDa) and β -galactosidase (116 kDa) molecular mass markers. Polyacrylamide gels were subjected to autoradiography with intensifying screens at -70°C for 3 h in (A) and for 3 days in (B).

A204 or R52 cells, suggesting a lack of a significant role for Arg¹⁶⁰ in the interaction between PDGF-BB and either receptor isotype.

To investigate whether the ability of PDGF-BB to activate PDGF receptors was similarly affected by disruption of the cationic region, the abilities of WT-RKK and RKK → EEE to induce receptor autophosphorylation *in vitro* were assessed in A204 and R52 cells. WT-RKK elicited dose-dependent autophosphorylation of 170 kDa PDGF receptor species in both A204 cells (Figure 2A) and R52 cells (Figure 2B). In A204 cells, however, the RKK → EEE mutant was much less potent than the wild type, with concentrations as high as 3 $\mu\text{g/ml}$ required to elicit detectable α -receptor autophosphorylation, whereas no autophosphorylation of β -receptors was obtained in R52 cells. These observations correlate with the decreased receptor binding

Figure 3 Analysis of mitogenic activity of loop III mutants of PDGF-BB: incorporation of [³H]thymidine into newly synthesized DNA

The mitogenic activities of *P. pastoris*-expressed PDGF-BB polypeptides [WT-RKK (□), RKK → EEE (○), RKK → SSS (●) and RKK → SKK (▼)] were assessed for their relative abilities to induce the incorporation of [³H]thymidine into DNA in Swiss 3T3 (NR6) cells. Dose-response curves are representative of two independent experiments.

activity of RKK → EEE, indicating that disruption of the RKK motif with negatively charged glutamate residues also interferes with its ability to activate both α - and β -receptors.

The receptor binding-defective mutant RKK → EEE is mitogenically active

Mitogenic activities of wild-type and mutant B-chain proteins expressed in *P. pastoris* were assessed by [³H]thymidine incorporation assay in NR6 cells, a variant of the Swiss 3T3 cell line that expresses primarily α -receptors. A steep dose-response curve was observed with WT-RKK (Figure 3), with a half-maximal effective dose (ED₅₀) of 52 ng/ml (Table 1). The RKK → SKK and RKK → SSS mutants exhibited slightly higher potencies than WT-RKK, whereas, surprisingly, the potency of the RKK → EEE mutant was identical with that of WT-RKK. To exclude the possibility of interference from potential contaminating mitogens in *P. pastoris* culture supernatants, WT-RKK and RKK → EEE were purified to apparent homogeneity before analysis: identical results were obtained (results not shown). Similar results were obtained in β -receptor-expressing R52 cells (results not shown). The ability of the receptor binding-defective mutant RKK → EEE to stimulate DNA synthesis fully suggests that PDGF-BB might stimulate the entry of cells into the S phase through a mechanism distinct from high-affinity binding to PDGF receptors.

Alteration of the RKK sequence interferes with binding of PDGF-BB to heparin

During initial attempts to purify the RKK → EEE mutant, a marked loss in binding to heparin-agarose was noted. Because of the physiologically important role of heparin-containing proteoglycans in the association of PDGF-BB and the so-called 'long' form of PDGF-AA with the extracellular matrix [20,21], binding of the loop III mutants to heparin was examined in further detail. To quantify the heparin-binding activity of these proteins, a heparin-agarose binding assay *in vitro* was developed. Employing

Figure 4 RKK → EEE mutation interferes with cell-surface binding of PDGF-BB: pulse-chase analysis

CHO cells stably expressing WT-RKK (A) and mutant RKK → EEE (B) B-chains were metabolically labelled with [³⁵S]cysteine for 30 min and chased with a 200-fold excess of unlabelled cysteine for the indicated times (0, 1, 3, 6 and 24 h). Cell-surface-associated ('cell surface', left section of panels) and secreted ('medium', right section of panels) PDGF-BB was assessed by the removal of supernatants at each chase time point and the treatment of cells for 10 min with fresh chase medium containing 1 mM suramin. Suramin washes and culture supernatants were subjected to immunoprecipitation with anti-(human PDGF-BB) serum and SDS/PAGE under non-reducing conditions. The WT-RKK autoradiogram was obtained from a 30-day exposure of the gel to X-ray film; the exposure of the RKK → EEE gel was for 11 days. Indicated in each panel are the relative mobilities of carbonic anhydrase (29 kDa) and β -lactoglobulin (18.4 kDa). Also indicated outside the panels with arrows are the predicted electrophoretic mobilities of the 34 and 35 kDa cell-surface-associated forms of PDGF-BB (p34/35) and the 29–32 kDa species of secreted PDGF-BB (p29–32).

buffer conditions that were near-physiological with respect to pH and NaCl concentration, nearly 100 % of WT-RKK bound to the matrix. However, binding of RKK → EEE and RKK → SSS were significantly impaired, with only 38 % of each protein appearing in the bound fraction. The RKK → SKK mutant also exhibited decreased heparin-binding (74 % bound), implicating Arg¹⁶⁰ in the binding interaction. Taken together, these results strongly suggest that the loop III region contributes to the heparin-binding interface of the PDGF-BB molecule.

RKK → EEE mutant is secreted as non-cell-associated species of 29–32 kDa

The decreased heparin-binding activity of RKK → EEE *in vitro* suggested that the protein might be altered in its association with heparan sulphate proteoglycans (HS-PGs) in the extracellular matrix. PDGF-BB has been shown to associate efficiently with the cell surface [22,23], probably via interactions with HS-PGs [24,25], and the cell-surface-associated form is releasable with the heparin-like polyanion suramin. Thus the kinetics of cell surface association and secretion of PDGF-BB were analysed by a pulse-chase approach in CHO cells expressing WT-RKK or RKK → EEE (Figure 4). As shown previously [11,22], WT-RKK appears on the cell surface within 1 h in a suramin-releasable form as a doublet of 34 and 35 kDa (p34/35) that peaks within 3 h and disappears by 24 h (Figure 4A, left panel). Only trace amounts of non-cell-associated WT-RKK (p29–32) were seen by 24 h of chase (Figure 4A, right panel). No significant change in suramin-releasable p34/35 was observed for the RKK → EEE mutant over the 24 h course of the experiment (Figure 4B, left panel). However, a slight increase was apparent in the amount of mutant p34/35 appearing in the culture medium at 6 and 24 h of chase, suggesting an impairment in cell-surface association. Moreover, a marked increase was seen in the release of p29–32 into the culture supernatant at 6 and 24 h, consistent with an important role of the RKK motif in stabilizing association with the cell surface.

Analysis of RKK → EEE mutant structure by CD spectroscopy

To confirm that the diminished receptor-binding and heparin-binding activities of the RKK → EEE mutant were not a function of major alterations in secondary structure, *Pichia*-expressed RKK → EEE was purified to homogeneity as described in the Methods and materials section and analysed by CD spectroscopy. The purity and molecular masses of recombinant protein preparations were assessed by SDS/PAGE under non-reducing and reducing (dimer-dissociating) conditions before CD analysis (Figure 5). In the absence of dithiothreitol, WT-RKK migrated as a complex of 28–30 kDa species, very similar to those expressed in CHO cells [22], with trace amounts of a 15 kDa species observed in the preparation. On reduction, the 28–30 kDa complex was converted into a predominant 15.5 kDa monomeric form. The pattern of RKK → EEE molecular-mass species observed under non-reduced and reduced conditions was nearly identical with that seen for WT-RKK, with the exception of a trace of a non-reducible 24 kDa contaminant and equal amounts

Figure 5 SDS/PAGE analysis of purified WT-RKK and RKK → EEE forms of human PDGF-BB, expressed in *P. pastoris*

The indicated amounts of each protein were applied to SDS/15%-polyacrylamide gels, separated by electrophoresis under non-reducing and reducing (100 mM dithiothreitol) conditions, and stained with silver. Indicated at the left of each panel are the electrophoretic mobilities of molecular-mass markers (in kDa): ovalbumin (43 kDa), carbonic anhydrase (29 kDa), β -lactoglobulin (18.4 kDa), lysozyme (14.3 kDa) and bovine trypsin inhibitor (6.2 kDa).

Figure 6 RKK → EEE mutation does not significantly affect the secondary structure of PDGF-BB, as assessed by far-UV CD spectroscopy

Displayed is the CD spectrum obtained for the RKK → EEE mutant of PDGF-BB (continuous curve) in relation to the spectrum previously reported for wild-type PDGF-BB (spiky trace) by Craig et al. ([26], by permission of the authors). Data are shown as molar ellipticities. Note the use of different y-axes bearing a slight difference in scale to demonstrate that the spectra of the two proteins are nearly identical in shape. The CD spectrum of the RKK → EEE mutant represents the average of eight scans and was recorded at 20 °C with a 0.1 cm path-length cell and a 2 nm bandwidth. The protein was analysed at 0.20 mg/ml in 10 mM acetic acid, pH 3.2.

of reduced p15.5 and p14 species. The appearance of the monomeric p14 species of RKK → EEE was possibly the result of increased proteolytic cleavage, secondary to the high yeast culture density and longer collection time before harvest.

The CD spectrum obtained with the RKK → EEE protein (Figure 6) was essentially identical with that reported for human PDGF-BB expressed in *S. cerevisiae* [26]. The spectrum was characterized by a significant shoulder at 228 nm and strongly positive ellipticity at 190 nm, both of which were consistent with a high percentage of β -sheet structure in the molecule, estimated to be approx. 50%. The centre of the minimum at 203 nm was indicative of high random-coil content (43%); the molecule possessed only a small percentage of α -helical residue content (7%). These secondary-structure estimates are also in close agreement with those obtained previously for PDGF-BB [26], as well as with the PDGF-BB structure derived directly by X-ray diffraction [7]. The lack of any detectable significant disruption in secondary structure for the RKK → EEE protein strongly suggests that the impairment in binding to receptors and heparin, as well as the increased secretion of the protein, were not the result of major structural perturbations in the molecule.

Loop III mutants of the PDGF A- and B-chains exhibit dominant-negative activity

The receptor binding-deficient RKK → EEE mutants of the PDGF A-chain [8] and B-chain remain able to dimerize, suggesting a potential for dominant-negative activity via inactivation of endogenous PDGF monomers. The human glioblastoma cell line U87 was employed for assessment of dominant-negative activity, because a PDGF autocrine loop drives its malignant growth, even in the absence of exogenous growth factors such as serum [19]. U87 cells were stably transfected with cDNA species encoding wild-type A- and B-chains, as well as their RKK → EEE mutant counterparts, as described in the

Figure 7 PDGF dominant-negative mutants decrease the tumorigenicity of human U87 glioblastoma cells in athymic nude mice

Exponentially growing cells (10^7) of the indicated U87 transfectants were inoculated subcutaneously behind the scapulae of athymic nude mice as described in the Materials and methods section. Each growth curve represents averaged data obtained for separate, stably transfected clones. For neomycin-resistant controls (panel labelled Neo), two clones, 3.1 (○) and 3.3 (●), were examined; data points shown in this panel are means $\pm$ S.E.M., with four mice injected per clone. For the remaining panels, the following clones were evaluated: A^{wt}, clones 5.1 (●, one replicate), 5.4 (○, two replicates) and 6.4 (□, two replicates); B^{wt}, clones 10.3 (●, two replicates), 10.1 (○, two replicates) and 9.3 (□, two replicates); A-chain/EEE, clones 8.4 (●, two replicates), 8.1 (○, two replicates) and 7.1 (□, one replicate); B-chain/EEE, clones 11.1 (●, one replicate), 12.1 (○, two replicates) and 12.5 (□, two replicates). The x-axis represents mean tumour volume calculated from the formula $V = (\pi/6)LW^2$, where L is the longest dimension and W the distance perpendicular to, and on the same plane as, L [40].

Materials and methods section. When growth characteristics of transfected U87 clones were assessed in cell culture, one of the A-chain RKK → EEE-expressing clones (clone 8.1; results not shown), exhibited a dependence on exogenous growth factor (i.e. fetal bovine serum) consistent with disruption of the PDGF autocrine loop by this construct.

Effects of RKK → EEE mutant expression on malignant growth of U87 cells were also evaluated in athymic nude mice. Neomycin-resistant controls all developed prominent tumours that were detected within approx. 7 days (Figure 7) and reached

average sizes of 1–4 cm³ within 20 days. Growth of tumours from clones overexpressing either A-WT-RKK or B-WT-RKK was not significantly different from that of neomycin-resistant controls. However, overexpression of either A-RKK → EEE or B-RKK → EEE almost totally curtailed tumour growth *in vivo*. In two out of three A-RKK → EEE clones examined (8.1 and 8.4), no tumour development was observed over a 30-day time course, whereas only small tumours (less than 1 cm³) could be observed by 40 days in the third clone (7.1). Similar results were obtained with the three B-RKK → EEE clones evaluated. Taken together, these results indicate dominant-negative activity of RKK → EEE mutants of the PDGF A- and B-chains, and support previous evidence [19] for a role of PDGF in mediating the growth of U87 cells *in vitro* and *in vivo*.

DISCUSSION

Conversion of the RKK sequence into three glutamic residues (RKK → EEE) resulted in a marked decrease in the ability to bind PDGF α and β receptors (Figure 1) and induce receptor autophosphorylation (Figure 2). Replacements of the RKK sequence with the polar, uncharged amino acid serine were not as disruptive to receptor binding as glutamic substitutions, although conversion of all three residues into serine resulted in a small decrease in binding to β -receptors and a significant decrease in the slope of the β -receptor binding curve. The ability of serine to substitute partly for the RKK sequence suggests that its hydroxyl moieties can form stabilizing bonds (i.e. hydrogen bonds) with receptor. The marked inhibitory effect of glutamate residues suggests an inability of this amino acid to form such bonds, or possibly an electrostatic repulsion with the receptor. Consistent with this notion is the observation that replacement of Lys¹⁶¹ with the uncharged, non-polar alanine residue impairs α -receptor binding by a factor of 4–10, whereas replacement with the negatively charged aspartic residue decreases binding to both α - and β -receptors by a factor of more than 10 [10]. Individual alanine substitutions at Arg¹⁶⁰ and Lys¹⁶² were without apparent effect in that study. These observations suggest that Lys¹⁶¹ might be the critical loop III residue involved in binding to both receptor isotypes, although the marked decrease in binding affinity observed in our study with three simultaneous glutamic substitutions also suggests possible contributions of Arg¹⁶⁰ and Lys¹⁶². Also relevant to our study is the recent report of the crystal structure of the structurally related vascular endothelial growth factor in complex with domain 2 of the Flt-1 receptor [27], in which amino acids of vascular endothelial growth factor (located between β -strands 5 and 6) corresponding to the loop III region of PDGF B-chain were shown to contribute significantly to the receptor-binding interface.

Previous studies have indicated that cationic amino acids encoded by the sixth exons of the PDGF A- and B-chains have an important role in the abilities of PDGF-AA and -BB to remain associated with the cell surface and extracellular matrix via binding interactions with HS-PGs [20,26]. This association is mediated by interactions with the heparin-like glycosaminoglycan moieties of the HS-PG [20,28–31] and is considered to involve electrostatic interactions between the cationic amino acids in the PDGFs and negatively charged groups of the polysaccharide. The biological importance of this interaction is indicated by the inhibition of PDGF-induced mitogenesis with either heparin-like glycosaminoglycans or cationic peptides derived from the region encoded by exon 6 of the A-chain [30,32]. The current study suggests, however, that regions other than the exon 6-encoded sequence, such as the loop III domain, contribute to the heparin-binding activity of the B-chain. The partial loss

of heparin binding observed with the RKK → SKK mutant indicates a specific role for Arg¹⁶⁰, whereas the more marked inhibition resulting from simultaneous substitutions at all three residues (RKK → EEE and RKK → SSS) strongly suggests that one or both of the remaining residues (Lys¹⁶¹ and Lys¹⁶²) also contribute to the binding interaction. The loop III region (Val¹⁵⁹-Arg¹⁶⁰-Lys¹⁶¹-Lys¹⁶²-Pro¹⁶³-Ile¹⁶⁴-Phe¹⁶⁵-Lys¹⁶⁶) is very similar in amino acid sequence to the consensus sequence for glycosaminoglycan recognition, -XBBBXXBX-, where B represents basic residues and X denotes uncharged residues [33]. The disruptive effect of serine substitutions within the cationic RKK sequence on heparin binding contrasts with the lack of effects on receptor binding and suggests the possibility of electrostatic interactions with heparin, although hydrogen bonding and van der Waals interactions have also been proposed to mediate such interactions in other proteins [33]. The contribution of the RKK sequence to heparin binding of the B-chain can also be contrasted with a much less pronounced role in the A-chain, where the RKK → EEE mutant exhibits avid heparin binding that is stable at NaCl concentrations greater than 1 M (results not shown). Nevertheless, the 'short form' of PDGF A-chain, which lacks the exon 6-encoded retention sequence, also exhibits diminished heparin-binding activity [34], suggesting that the RKK sequence or perhaps other sequences might provide a limited contribution.

A role of the RKK sequence in mediating binding between the cell-associated 34/35 kDa species and the extracellular matrix is supported by pulse-chase analyses of CHO cells that express the RKK → EEE mutant. The accumulation of the 29–32 kDa species of RKK → EEE, and to a smaller extent the 34/35 kDa species in the culture supernatant, suggests a lower affinity of the mutant for extracellular matrix. These results indicate that the previously characterized C-terminal retention domain of the B-chain, which remained intact in the 34/35 kDa species of the RKK → EEE mutant, is insufficient to mediate full association of PDGF-BB with the cell surface and extracellular matrix. Taken together, the heparin binding and pulse-chase studies *in vitro* suggest that the heparin-binding interface of the PDGF-BB molecule is composed of cationic amino acid residues found in both the loop III and C-terminal regions.

Sequestration of growth factors via binding to HS-PGs in the extracellular space has been proposed [35] to serve numerous functions, such as protecting against proteolytic degradation, restricting the range of growth factor action and serving as a reservoir for sustained activity after initial release. Our observations indicate that the RKK sequence has an important role in maintaining PDGF-BB in a sequestered state after secretion. In addition, the participation of the RKK sequence in binding to both receptor and heparin is intriguing and suggests that HS-PG binding might block interactions with receptor.

The lack of impairment in mitogenic activity of the receptor binding-deficient RKK → EEE mutant was unexpected, especially because the analogous RKK → EEE mutant of the A-chain is equally impaired in its receptor binding and mitogenic activities [8]. Dissociation of binding and mitogenesis has a precedent, however, in studies of PDGF and other growth factors. For example, Clements et al. [36] demonstrated that substitution for Arg¹⁰⁸ with glutamate in the PDGF B-chain significantly impaired its ability to bind receptor (by a factor of approx. 10) and to stimulate inositol lipid turnover (by a factor of more than 35), but did not affect mitogenic activity. In addition, a recent study has demonstrated that fibroblast growth factor (FGF) can induce mitogenic signalling in the presence of high concentrations of heparin that interfere with high-affinity receptor binding [37]. How can mitogenic signalling be elicited by growth factors without high-affinity binding to receptors? A

PDGF receptor-independent pathway for mitogenic signalling is unlikely, because many studies have demonstrated the necessity of receptor tyrosine kinases for mitogenesis induced by PDGF [38], FGF [37] and other ligands. A second possibility, that the RKK $\rightarrow$ EEE protein can fully stimulate mitogenesis through a minimal degree of receptor occupancy, also seems improbable because the ED₅₀ for mitogenesis of the mutant is unaltered. In a study of FGF/FGF-receptor interactions, Nugent and Edelman [39] have proposed a model termed 'transient receptor binding' in which a low-affinity ligand can elicit mitogenic signalling if the on-rate of binding is significant. Detailed kinetic measurements of on-rates and off-rates of wild-type and mutant PDGFs will be required to examine this model further as an explanation for the full mitogenic signalling activity of the receptor binding-deficient mutant protein.

Overexpression of PDGF A- and B-chains harbouring the RKK $\rightarrow$ EEE mutation inhibited the formation of foci of U87 astrocytoma cells in culture, with the A-chain RKK $\rightarrow$ EEE mutant conferring a dependence on exogenous serum growth factors for growth. The growth inhibitory activities of both the A-chain and B-chain RKK $\rightarrow$ EEE mutants on U87 tumours in athymic nude mice suggest that high-affinity binding, mediated in part by interaction between the RKK domain and PDGF receptor, is required for maintenance of the malignant phenotype of these cells. The efficacy of these and other dominant-negative PDGF mutants [15,19,40] suggest possible avenues for gene therapy approaches to astrocytoma and other cancers in which autocrine and paracrine activities of PDGF might have a role.

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