Purification and analysis of proteinase-resistant mutants of recombinant platelet-derived growth factor-BB exhibiting improved biological activity

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Recombinant platelet-derived growth factor (PDGF)-BB was expressed and secreted from yeast in order to study the structure–function relationships of this mitogen. A simple purification scheme has been developed which yields greater than 95% pure PDGF-BB. Analysis of this recombinant PDGF-BB shows partial proteolysis after arginine-32. Substitution of this arginine residue, or arginine-28 [a potential KEX2 (lysine–arginine endopeptidase) cleavage site], prevents or reduces cleavage of PDGF-BB respectively. These mutations result in a 5-fold increase in expression levels of PDGF-BB, and the resulting mutant proteins show higher activity in a number of biological assays than the cleaved wild-type PDGF-BB. These data are in accord with previous work by Giese, LaRochelle, May-Siroff, Robbins & Aaronson [(1990) *Mol. Cell Biol.* 10, 5496–5501] suggesting that the region isoleucine-25–phenylalanine-37 is involved in PDGF-receptor binding.

INTRODUCTION

Platelet-derived growth factor (PDGF) is a mitogen released from the α -granules of platelets after activation (Kaplan *et al.*, 1979; Ross *et al.*, 1986). This growth factor can also be produced by a number of other cell types, such as cultured vascular smooth-muscle cells, bovine aortic endothelial cells and monocytes/macrophages (DiCorleto & Bowen-Pope, 1983; Ross *et al.*, 1986). The mitogenic activity of this growth factor is restricted to cells of mesothelial origin, including smooth-muscle cells, glial cells and fibroblasts, but excluding arterial endothelium (Ross *et al.*, 1974; Ross & Vogel, 1978). PDGF has a number of additional biological functions, including chemotaxis for monocytes and neutrophils (Deuel *et al.*, 1982).

PDGF isolated from blood is predominantly a disulphide-linked homodimer or heterodimer of M_r 28 000–31 000 composed of two chains, A and B (Heldin *et al.*, 1979; Raines & Ross, 1982). The mature A- and B-chains of PDGF show sequence identity at 51% of residues, and the eight cysteine residues in each chain are conserved (Johnsson *et al.*, 1984). The PDGF B-chain is almost identical in sequence with the v-sis oncogene product, p28^{sis}, derived from the simian-sarcoma virus (Doolittle *et al.*, 1983; Waterfield *et al.*, 1983; Johnsson *et al.*, 1984). The major form of PDGF found in human serum is a heterodimer of A- and B-chains, but homodimers are also present in small quantities (Hammacher *et al.*, 1988a; Hart *et al.*, 1990). Heterodimers are preferentially formed in the presence of both A- and B-chains (Ostman *et al.*, 1988; Hoppe *et al.*, 1990). Homodimers of PDGF-BB are found in porcine serum (Stroobant & Waterfield, 1984), and PDGF-AA is produced by several human tumour-cell lines (Heldin *et al.*, 1986; Hammacher *et al.*, 1988b).

Recent work has shown that two types of PDGF receptor exist: α and β (Hammacher *et al.*, 1989; Seifert *et al.*, 1989). The β -receptor can only bind the B-chain of PDGF, but the α -receptor binds both A- and B-chains of PDGF. Thus different

biological functions of PDGF are likely to be dependent on the proportion of α - and β -receptors on cells (Hart *et al.*, 1988; Nister *et al.*, 1988; Hammacher *et al.*, 1989; Seifert *et al.*, 1989).

Much of the recent work on PDGF has been performed using recombinant material derived from mammalian cells (Ostman *et al.*, 1988), yeast (Kelly *et al.*, 1985; Ostman *et al.*, 1989) or *Escherichia coli* (Hoppe *et al.*, 1990). Both A- and B-chains undergo proteolytic processing during expression and secretion *in vivo*, resulting in mature chains of 104 amino acids for the A-chain (Johnsson *et al.*, 1984) and 109 amino acids for the B-chain (Johnsson *et al.*, 1984; Josephs *et al.*, 1984). In addition, recombinant PDGF secreted by yeast shows partial cleavage of the B-chain between Arg-32 and Thr-33 and the full-length A-chain at the Lys-11–Thr-12 bond (Ostman *et al.*, 1989). N-Terminal processing of the PDGF B-chain, at Leu-5–Thr-6 and Arg-32–Thr-33, has also been observed in preparations from human platelets, but no processing of the A-chain was identified (Hart *et al.*, 1990). No N-terminal processing or internal cleavage was observed in PDGF-AB secreted by transfected Chinese-hamster ovary cells (Ostman *et al.*, 1988) or PDGF-AA from baby-hamster kidney cells (Eichner *et al.*, 1989). The effect of partial cleavage on the activity of the molecule has not been reported previously. Here we describe the production and biological activity of PDGF-BB mutants that show no internal cleavage.

MATERIALS AND METHODS

Construction of a synthetic gene and mutants

A gene encoding human PDGF-B with a hybrid codon usage optimized for expression in yeast was constructed by the British Bio-technology Ltd. Laboratory Products Division and cloned on a HindIII–EcoRI restriction fragment into pUC18 (Yanisch-Perron *et al.*, 1985). The gene was adapted for fusion to the yeast alpha factor pre-pro sequence by digesting the plasmid with

Abbreviations used: CM, carboxymethyl; DMEM, Dulbecco's modified Eagle's medium; FCS, foetal-calf serum; HRP, horseradish peroxidase; PBS, phosphate-buffered saline (8.1 mM- Na_2HPO_4 /1.5 mM- KH_2PO_4 /0.137 M- NaCl /0.003 M- KCl , pH 7.3); PDGF, platelet-derived growth factor; ED_{50} , concentration at which 50% of maximal stimulation is obtained.

HindIII-HpaI, purifying the vector sequence and cloning into the plasmid an oligonucleotide linker coding for the fusion junction between alpha factor and PDGF-B. A truncated PDGF-B gene (1–109) was designed to produce material equivalent to the mature PDGF-B chain. The truncated PDGF-B gene and mutants [Ser²⁸]PDGF-B and [Pro³²]PDGF-B were created by oligonucleotide site-directed mutagenesis of the cloned PDGF-B gene in M13 using the method described by Kunkel *et al.* (1987). After mutagenesis, the genes encoding PDGF-B and its variants were cloned as a *HindIII-BamHI* fragment into the yeast expression vector pSW6.

Expression in yeast

Plasmid pSW6 contains an *E. coli* replication origin and an ampicillin-resistance gene derived from pUC18 (Yanisch-Perron *et al.*, 1985). The yeast replication origin and *LEU2-d* gene are derived from the plasmid pJDB219 (Beggs, 1978). Transcription of PDGF-B is controlled by a hybrid yeast PGK and *GAL1-10* promoter. The PDGF coding sequence is fused in-frame with the pre-pro sequence of the yeast-alpha-factor gene (Kurjan & Herskowitz 1982) at the *HindIII* restriction site. The minimal PGK promoter vector was as described by Chambers *et al.* (1989), with the exception that a 140-bp *GAL1-10* UAS *RsaI-AluI* fragment (West *et al.*, 1984) was cloned into the *BamHI* site of pKV560 and interferon sequences replaced by PDGF-B. Plasmids were transformed into yeast *Saccharomyces cerevisiae* strain BJ2168 (a *prc1-407 prb1-1122 pep4-3 leu2 trp1 ura3-52*; Yeast Genetic Stock Center, University of California, Berkeley, CA, U.S.A.) by the method of Beggs (1978). All yeast media were as described by Sherman *et al.* (1986). For expression experiments, yeast were grown in 1% (w/v) glucose/synthetic complete medium without leucine to an A_{600} of 4 and the cells collected by centrifugation. The cells were then inoculated into twice the original culture volume of 1% (w/v) galactose/0.2% (w/v) glucose/synthetic complete medium without leucine and grown at 30 °C with shaking in baffled flasks.

For measurement of intracellular concentrations of PDGF-BB, 10 ml of cells were centrifuged and resuspended in 0.5 ml of 0.9 M-sorbitol / 20 mM-EDTA / 50 mM-2-mercaptoethanol / Zymolyase 20T (20 μ l of a 100 mg/ml solution; Seikagaku Kogyo, Tokyo, Japan). The cells were incubated at 30 °C for 1 h, then centrifuged and the supernatant removed and assayed by e.l.i.s.a. for the PDGF-BB concentration in the yeast cell wall. The cells were washed in 1 ml of 1.0 M-sorbitol and collected by centrifugation. To lyse the cells, the pellet was resuspended vigorously in 0.1 M-NaCl/0.1% (w/v) SDS/0.1 M-Tris/HCl, pH 7.5, and lysis was monitored under the microscope. The debris was removed by centrifugation and the supernatant assayed by e.l.i.s.a. for the intracellular concentration of PDGF-BB.

Transcript and copy-number analysis

RNA was prepared from yeast strains grown in glucose (uninduced) or in galactose (induced) as described by Clements *et al.* (1989). Total RNA was separated in a 1.5% (w/v)-agarose gel containing formaldehyde and transferred to a nitrocellulose filter (Thomas, 1980). The filter was baked and then probed with [γ -³²P] oligonucleotides corresponding to PDGF and the yeast ribosomal RNA which was included as an internal loading standard. Hybridization conditions were as described by Thomas (1980), except that the hybridizations were carried out at 20 °C for 16 h and the filter washed at 20 °C in several changes of 0.9 M-NaCl/0.9 M-NaCl/0.09 M-sodium citrate/0.1% (w/v) SDS.

Purification of PDGF-BB

The purification scheme is a modification of the method of

Raines & Ross (1982), which describes purification of PDGF from human platelets. Yeast supernatant (5–6 litres) was clarified by centrifugation at 8600 *g* for 20 min at 4 °C. The pH was adjusted to 6.0 with 20 mM-Tris before 5–10-fold concentration by tangential-flow ultrafiltration (Millipore Minitan; PTGC 10000 membranes). The molarity of the concentrated supernatant was adjusted to 0.09 M with NaCl before chromatography on a 200 ml CM-Sepharose column (Pharmacia; 5 cm $\times$ 10 cm). The column was washed with 2–3 vol. of 0.19 M-NaCl/20 mM-Tris/HCl, pH 6.0, and fractions containing PDGF-BB were eluted with 1 M-NaCl/20 mM-Tris/HCl, pH 7.4. The eluate was applied directly to a 20 ml phenyl-Sepharose column (Pharmacia, 1.6 cm $\times$ 10 cm) equilibrated in the same buffer, followed by washing with 60 ml of 1 M-NaCl/20 mM-Tris/HCl, pH 7.4. PDGF-BB was eluted with 40–60 ml of 50% (v/v) ethylene glycol/0.15 M-NaCl/20 mM-Tris/HCl, pH 7.4. All chromatography steps were performed at 4 °C. Fractions containing PDGF, assessed by e.l.i.s.a. or mitogenic assay, were pooled and stored at –20 °C for up to 3 months.

Size-exclusion chromatography

For measurement of protein by absorbance at 280 nm and *N*-terminal sequencing, PDGF was transferred from phenyl-Sepharose elution buffer into 10 mM-acetic acid by using a two-step size-exclusion chromatography process. The first size-exclusion-chromatography step, using a 250 ml Sephadex G-25M column (Pharmacia; 5 cm $\times$ 12.5 cm), transfers PDGF into 0.5 M-urea/10 mM-glycine/HCl, pH 3.0. Protein fractions from this column were pooled and applied to a further 250 ml Sephadex G-25M column and eluted with 10 mM-acetic acid. Fractions containing PDGF-BB were pooled, concentrated by freeze-drying and stored at –20 °C.

The protein concentration was determined by A_{280} . The specific absorption coefficient for PDGF-BB was calculated as 0.46 litre $\cdot$ g^{–1} $\cdot$ cm^{–1}, from the content of aromatic residues and summation of residue absorbances (Scopes, 1974).

SDS/PAGE

Samples from chromatography columns were dialysed against 0.1% (w/v) SDS or transferred into 10 mM-acetic acid and freeze-dried before analysis by SDS/PAGE. Electrophoresis was performed using 4% stacking gels and 15% separating gels (Mini-Protean II; Bio-Rad), at 200 V for approx. 45 min at 20 °C (Laemmli, 1970). Analytical samples (0.5–2.0 μ g per track) were separated on 8–25% Phast gels (Pharmacia) for 75–90 V $\cdot$ h at 20 °C, under either non-reducing or reducing conditions (0.7 M-2-mercaptoethanol). Protein was revealed with either Coomassie Brilliant Blue R250 (BDH) or by a silver-staining technique (See & Jackowski, 1989).

Western blotting

Electrophoresis was performed as described above and separated proteins were transferred on to nitrocellulose sheets (Schleicher & Schuell) by the method of Towbin *et al.* (1979), at 100 V for 1 h at 4 °C (Mini Trans Blot, Bio-Rad). Non-specific binding was blocked by incubation of the nitrocellulose sheets with 2% (w/v) BSA/phosphate-buffered saline (PBS; Dulbecco A, Oxoid) for 16 h at 4 °C. PDGF was detected by incubation with a 1:200 dilution of rabbit anti-(human PDGF) antibody (R & D systems, Minneapolis, MN, U.S.A.) in PBS for 1 h at 20 °C. Excess antibody was removed by washing three times with 0.5% (v/v) Nonidet P40/PBS and was followed by incubation with 1:1000 dilution of horseradish peroxidase (HRP)-conjugated goat anti-(rabbit IgG) serum (Sigma) for 1 h at 20 °C. The nitrocellulose sheets were finally washed twice with 0.5% Nonidet

Fig. 1. Sequence of the synthetic PDGF-B gene

The sequence has been modified at the 5' end to allow the in-frame fusion to the pre-pro sequence of alpha-factor. The amino acid sequence corresponding to PDGF-B is shown above the DNA sequence. The sequences removed to create truncated PDGF-B are indicated by the boxed region. The positions of [Ser²⁸]PDGF-BB and [Pro³²]PDGF-BB mutations are indicated. The restriction sites used to clone PDGF-B into pSW6 are shown.

P40/PBS and twice with PBS. The blots were developed with 0.05 % (w/v) diaminobenzidine/0.03 % (v/v) H₂O₂/PBS.

N-Terminal sequencing

N-Terminal sequence data was obtained with a 471A Applied Biosystems pulsed liquid sequenator, with a liquid-chromatography system modified to use a 140A solvent delivery system. A portion (8 µg) of PDGF-BB, [Ser²⁸]PDGF-BB or [Pro³²]PDGF-BB in 0.1 % (v/v) trifluoroacetic acid or 10 mM-acetic acid were subjected to 12 cycles of automated Edman degradation. The amino acids detected were aligned with the PDGF-B sequence and the percentage of each N-terminal amino acid calculated. Internal cleavage at Thr-33 was expressed as the percentage of total PDGF, independent of the small amount of N-terminal processing from Ser-1 to Thr-6, since this is likely to occur in both cleaved and uncleaved forms.

Cell culture

Swiss 3T3 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10 % (v/v) foetal-calf serum (FCS) at 37 °C in a humidified atmosphere of CO₂/air

(1:19). Cultures were routinely monitored for spontaneous transformants.

Mitogenesis assay

The uptake of [³H]thymidine by Swiss 3T3 cells was used as a measure of DNA synthesis. Cells were seeded into microtitre plates (Falcon) at a density of 1 × 10⁴ per well with DMEM containing 10 % FCS. After incubation at 37 °C for 6–8 days, when the cells were confluent and quiescent, diluted test samples (yeast supernatant or purified material) were added directly to the wells. The plates were incubated for 16 h at 37 °C before pulsing with [³H]thymidine (1 µCi/ml) for 6 h. Incorporation of [³H]thymidine into cellular material insoluble in trichloroacetic acid was determined as described by Raines & Ross (1985). Serial dilutions of a PDGF-BB standard, c-sis (Amersham), from 20 ng/ml to 0.1 ng/ml were used to obtain a dose-response curve. The concentration of PDGF in unknown samples is calculated by multiplying the dilution of unknown which produces 50 % of the maximal response by the ED₅₀ (the concentration at which 50 % of the maximal stimulation is obtained) of the c-sis standard. For comparison of biological activities of the purified proteins (PDGF-BB, [Ser²⁸]PDGF-BB and [Pro³²]PDGF-BB mutants), stock solutions of known concentration (100 µg/ml; determined by A₂₈₀) were prepared, dose-response curves constructed and ED₅₀ values calculated.

Inositol-lipid-turnover assay

Swiss 3T3 cells were seeded into 24-well tissue-culture plates (Falcon) at 1 × 10⁶ per well. After 3 days, the cells were pre-labelled for a period of 72 h by adding [³H]myoinositol at 3 µCi/ml directly to the growth medium. The monolayers were rinsed and subsequently incubated for 20 min at 37 °C with 0.5 ml (per well) of Hanks Balanced Salt Solution containing 10 mM-LiCl to inactivate inositol-1-phosphatase (Berridge *et al.*, 1982). Dilutions of the samples and PDGF standard (c-sis made up at 25 times the desired concentration) were added to the wells and incubated at 37 °C for 20 min. Controls, such as yeast supernatant without PDGF, were included for comparison.

The reaction was stopped by aspiration of the medium and addition of 1 ml of ice-cold 5 % (v/v) perchloric acid per well. The plates were kept on ice for 30 min to extract the cellular inositol phosphates. Precipitated cellular protein was removed by centrifugation and perchloric acid quantitatively removed from the supernatant solution by the method of Khym (1975). Samples of the neutralized cell extracts (0.7 ml) were diluted to 5 ml with 5 mM-potassium tetraborate/0.5 mM-EDTA. The accumulated labelled inositol monophosphates were separated by the method of Bone *et al.* (1984) using AG 1-X8 anion-exchange resin (Bio-Rad). Column fractions were mixed with Optiphase scintillant (LKB) and the level of radioactivity determined for each fraction (Beckman LS 5000CE).

Solid-phase sandwich e.l.i.s.a.

This assay was developed using two antibodies to PDGF: goat anti-(human PDGF-AB) antibody (Collaborative Research) and rabbit anti-(human PDGF-BB) antibody (Genzyme). All washes and dilutions were made using 0.05 % (v/v) Tween 20 (Sigma)/PBS, unless stated otherwise.

A 96-well immunoplate (Nunc-Maxisorp) was coated overnight at 4 °C with goat anti-PDGF antibody at 5 µg/ml in 0.05 M-sodium carbonate/bicarbonate buffer, pH 9.6. Remaining protein adsorption sites were blocked by incubation with 0.1 % (w/v) casein/PBS for 30 min at 20 °C, and the plate was washed three times. Serial dilutions of the test samples and PDGF standard (c-sis; Amersham) were made by titration across the sensitized plate in PBS/Tween. The plate was incubated for 1 h

Fig. 2. Plasmid map of pSW6 and pJC75

The position of the expression cassette and genes involved in the replication and selection of the plasmid in yeast and *E. coli* are indicated. Key restriction sites are shown on the outside of the plasmids. Abbreviations: Amp, ampicillin-resistance gene; Ori, origin; GAL UAS, GAL upstream activation sequence; PGK term, PGK transcription terminator; 5' TRP 1, DNA 5' of *TRP1* gene; '2 µm' refers to yeast 2 µm circle DNA.

at 20 °C. After washing three times, 50 µl of rabbit anti-PDGF antibody (5 µg/ml) was added to each well and incubated for 1 h at 20 °C. The plate was washed for a further three times, followed by addition of HRP-conjugated goat anti-(rabbit IgG) serum (Tago), at a dilution of 1:1000, for 1 h at 20 °C. After five washes the chromogenic peroxidase substrate tetramethylbenzidine was added at 100 µg/ml in 0.1 M-sodium acetate buffered to pH 6.0 with 0.1 M citric acid/0.04 % (v/v) H₂O₂. When sufficient colour had developed (typically 5–15 min), the reaction was stopped by addition of 15 µl of 2.5 M-H₂SO₄/well, and the A₄₅₀ was read (Dynatech MR650 plate reader).

RESULTS

Construction of a synthetic gene

A synthetic gene for PDGF-B was designed on the basis of the published cDNA sequence of mature PDGF-B (Johnsson *et al.*, 1984). The codon usage was optimized to that of the yeast *Saccharomyces cerevisiae* (Bennetzen & Hall, 1982), and a number of useful restriction sites engineered into the sequence. The sequence codes for a 160-amino-acid-protein, starting at the *N*-terminal sequence of mature PDGF-B (Ser-Leu-Gly-Ser-Leu...) and ending at the natural translational stop codon (Fig. 1). A PDGF-B gene with the mature *C*-terminus was made by deleting

the sequence from the threonine residue at position 109 to the translational stop codon by oligonucleotide mutagenesis (Fig. 1). The *N*-terminal sequence of the synthetic genes was modified by the addition of an oligonucleotide linker to enable the fusion of the mature sequence to that of the yeast pre-pro-alpha-factor gene (Fig. 1) (Kurjan & Herskowitz, 1982). The alpha-factor sequence facilitates secretion from yeast, and mature PDGF-B protein is liberated from the pre-pro sequence by the action of the yeast KEX2 lysine-arginine endopeptidase (Julius *et al.*, 1984).

Expression in yeast

All expression experiments were carried out in the proteinase-deficient *S. cerevisiae* strain BJ2168. The genes encoding PDGF-B (residues 1–160) and PDGF-B (residues 1–109) were cloned into the yeast expression vector pSW6 (Fig. 2), in-frame with the pre-pro sequence of the yeast alpha-factor gene (Kurjan & Herskowitz, 1982). Transcription of PDGF in this plasmid is induced upon growth on galactose by a hybrid GAL1/PGK promoter. Mitogenic assay of the yeast culture medium confirmed the presence of biologically active PDGF-BB (Table 1). The presence of full-length and truncated PDGF-BB in culture medium was identified by Western blot. Two species of *M_r* 42000 and 32000 were identified (Fig. 3), values that are higher than those expected (32400 and 24600). No significant difference in expression levels or biological activity (Table 1) was observed between the full-length or truncated versions of PDGF-BB, confirming that the active portion of the molecule resides in the first 1–109 residues. Subsequent work was therefore carried out on the truncated PDGF-BB.

PDGF-BB expression levels were about 10-fold lower than our experience with other heterologous proteins such as epidermal growth factor (J. M. Clements, unpublished work) and maximum levels were only observed 6–7 days after induction. To determine the level at which expression of PDGF-BB was being limited, the plasmid copy number, transcript levels and the intra- and extracellular concentrations of PDGF-BB were determined after induction. The copy number of both pSW6/PDGF-B (1–160) and PDGF-B (1–109) were determined and found to be approx. 200 copies per cell. In addition, PDGF-B mRNA levels 16 h after transfer to galactose showed the expected induction of PDGF transcripts (Fig. 4). Intracellular levels of soluble PDGF-BB

Table 1. Expression levels of PDGF-BB, [Ser²⁸]PDGF-BB and [Pro³²]PDGF-BB in yeast supernatant

Expression levels for full-length (1–160) PDGF-BB, truncated (1–109) PDGF-BB and mutants [Ser²⁸]PDGF-BB and [Pro³²]PDGF-BB in pSW6 are shown. Yeast expressing PDGF-BB was grown for 6 days after induction and the supernatants were assayed by e.l.i.s.a. and mitogenic assay.

Gene product	Expression (µg of product/ml)	
	E.l.i.s.a.	Mitogenesis
PDGF-B (1–160)	0.334	0.309
PDGF-B (1–109)	0.468	0.354
[Ser ²⁸]PDGF-B	2.720	1.612
[Pro ³²]PDGF-B	2.960	2.020

Fig. 3. Western-blot analysis of PDGF-BB from yeast

Truncated PDGF-BB (lanes 1 and 2) and full-length PDGF-BB (lanes 3–6) isolated from yeast supernatant at day 3 (lanes 2, 4 and 6) or day 6 (lanes 1, 3 and 5) after induction, detected by Western blotting. Human platelet-derived PDGF-AB was included for comparison (lane 7). M_r standards (Bio-Rad prestained SDS/PAGE standards, low range) are in lane 8. Full-length PDGF-BB (1–160) is detected at M_r 42 000 and truncated PDGF-BB (1–109) at M_r 32 000.

Fig. 4. Transcript analysis of pSW6/PDGF-B (1–160) and pSW6/PDGF-B (1–109)

pSW6 (lanes 1 and 4), pSW6/PDGF-B (1–160) (lanes 2–5) and pSW6/PDGF-B (1–109) (lanes 3 and 6) were grown in 1% glucose/synthetic complete medium, the cells harvested, transferred to 1% galactose/synthetic complete medium and grown for 16 h. RNA was prepared from the yeast grown in glucose (lanes 1–3) and galactose (lanes 4–6). The RNA was analysed by Northern blot using 32 P-labelled oligonucleotides complementary to the synthetic PDGF-B gene mRNA and as an internal loading standard to the yeast 18 S mRNA.

Table 2. Purification of PDGF-BB from yeast supernatant

Purification step	Volume (ml)	Mitogenic activity (μ g/ml)	PDGF (mg)	Recovery (%)
Yeast supernatant	5500	0.62	3.41	100
Concentrated supernatant	750	3.88	2.91	85
CM-Sepharose and phenyl-Sepharose	55	39.82	2.19	64
2 $\times$ Sephadex G-25M	70	16.28	1.14	33

were detected 1 day after induction at approx. 0.6μ g/ml and remained relatively high over 6 days. However, it took 6 days for significant quantities of PDGF-BB to accumulate in the medium

Fig. 5. Purified PDGF-BB separated by SDS/PAGE

Purified PDGF was dialysed against 1% SDS or transferred into 10 mM-acetic acid, freeze-dried and redissolved in gel sample buffer (without 2-mercaptoethanol). Samples were applied to 15% polyacrylamide gels as follows: lane 1, M_r standards (Pharmacia low-molecular-mass calibration kit); lane 2, concentrated yeast supernatant; lane 3, CM-Sepharose non-absorbed fraction; lane 4, CM-Sepharose wash; lane 5, CM-Sepharose eluate; lane 6, phenyl-Sepharose non-absorbed fraction; lane 7, phenyl-Sepharose wash; lane 8, phenyl-Sepharose eluate. Electrophoresis was performed at 200 V for approx. 45 min, and protein was revealed by silver staining. Purified PDGF-BB (1–109) appears as a broad band at M_r 32 000.

(0.3μ g/ml) and, even at this stage, there was still 2–3 times more intracellular PDGF-BB. At no time was any significant level (< 4 ng/ml) of PDGF-BB found in the cell wall of yeast. A major limiting factor to PDGF-BB expression in yeast is, therefore, the secretion of PDGF-BB into the medium. This would indicate that the majority of PDGF-BB found in the culture medium is derived from limited cell lysis rather than true secretion.

As the expression levels were relatively low during the course of the work, expression of PDGF-B (1–109) was attempted from an alternative vector, pJC75, which contains a hybrid GAL1 upstream activation sequence with a minimal PGK promoter (Chambers *et al.*, 1989). Expression levels with this construct were consistently 50–100% higher than pSW6, and all further work was continued with this vector. It is thought that increased transcription from this vector accounts for the improved expression (D. R. Green & J. M. Clements, unpublished results).

Proteinase-resistant mutants

As part of a structure–activity relationship study of PDGF-BB, two PDGF-B mutants were constructed in which the equivalent PDGF-A residue was substituted for an arginine residue. Mutant [Ser²⁸]PDGF-BB disrupts one of the preferred cleavage sites (Arg–Arg) of the yeast KEX2 proteinase (Julius *et al.*, 1984). The second mutant, [Pro³²]PDGF-BB, disrupts one of the major proteinase cleavage sites in PDGF-BB between Arg-32 and Thr-33 (Ostman *et al.*, 1989; Hart *et al.*, 1990). Both the [Ser²⁸]PDGF-BB and [Pro³²]PDGF-BB mutants were expressed at levels approximately 5-fold higher than the wild-type molecule (Table 1). Analysis of the PDGF levels after induction revealed high intracellular levels for both mutants (0.4 – 1.5μ g/ml), but a much higher proportion of both mutants accumulated in the medium over 6 days than with PDGF-BB (Table 1). Thus the cleaved molecule may not be as efficiently secreted as the intact molecule; however, confirmation of this would require pulse-

Fig. 6. SDS/PAGE of PDGF-BB and mutants [Ser²⁸]PDGF-BB and [Pro³²]PDGF-BB under non-reducing or reducing conditions

Purified PDGF-BB (a), [Ser²⁸]PDGF-BB (b), or [Pro³²]PDGF-BB (c), were separated by SDS/PAGE on 8–25% Phast gels, under non-reducing (NR) or reducing (R) conditions (lanes 3 and 4). Proteins were visualized using Coomassie Blue stain. M_r standards in the range 16000–110000 (BioRad prestained SDS/PAGE standards, low range) are shown in lane 1 (a, b, c, d), lane 5 (c) and lane 6 (d) and M_r standards of 2500–16900 (LKB molecular weight markers) are in lane 2 (c, d), lane 5 (d) and lane 6 (a, b). The remaining lanes are blank. PDGF-BB is cleaved after Arg-32 and appears as a doublet under reducing conditions. c-sis (Amersham) was analysed under similar conditions (d) and appears heterogeneous under both non-reducing and reducing conditions.

chase experiments to determine the intra- and extra-cellular half-life of PDGF.

Purification of PDGF-BB

Substantial purification of PDGF-BB was achieved by sequential chromatography of concentrated yeast supernatant using CM-Sepharose and phenyl-Sepharose. The recovery of material after purification was estimated as 64% by mitogenic assay (Table 2). This material appears more than 95% pure on the basis of either Coomassie Blue or silver staining of gels (Fig. 5). The recovery of PDGF-BB after CM-Sepharose chromatography was improved from 60% at pH 7.4 to a yield of 82% at pH 6.0. Elution of PDGF-BB from CM-Sepharose could be achieved using either 0.5 M-NaCl or 1.0 M-NaCl/20 mM-Tris/HCl, pH 7.4. Some additional low- M_r contaminants were eluted from CM-Sepharose with 1.0 M-NaCl, but these were removed by phenyl-Sepharose chromatography (Fig. 5). The yield of PDGF from phenyl-Sepharose was found to be related to the concentration of ethylene glycol. A maximum recovery of 80% of active material was obtained using 50% ethylene glycol.

PDGF-BB can be transferred from ethylene glycol into 10 mM-acetic acid by a two-stage chromatography process. However, direct transfer from a hydrophobic environment into an aqueous environment resulted in aggregation of PDGF, assessed by light scattering at 320 nm. Introduction of an intermediate step in 0.5 M urea/10 mM-glycine/HCl, pH 3.0, before final transfer into

10 mM-acetic acid, reduces aggregation to less than 10%. Losses are greatest at this stage (Table 2), with recovery of only 40–50% of the material applied to the Sephadex G-25M columns.

Mutants [Ser²⁸]PDGF-BB and [Pro³²]PDGF-BB were purified to > 95% by using the same procedure, as shown by SDS/PAGE (Fig. 6). The yield of both mutants was greater than that for PDGF-BB, with 70–80% recovery after CM-Sepharose and phenyl-Sepharose chromatography. The improvement in recovery may be related to the higher concentrations of mutant proteins in the initial yeast supernatants, since recovery of PDGF-BB from CM-Sepharose has been found to be dependent on the concentration of PDGF in the loading sample (results not shown).

Analysis of PDGF by SDS/PAGE and N-terminal sequencing

PDGF-BB (1–109) appears as a single diffuse band of M_r 28000 by SDS/PAGE under non-reducing conditions. The calculated M_r for this dimer is 24600, and this discrepancy is thought to be due to glycosylation of the molecules. Deglycosylation of PDGF using trifluoromethanesulphonic acid (Edge *et al.*, 1981) yields a molecule of approx. M_r 26000 (results not shown), and carbohydrate can be detected both in PDGF-BB and in [Ser²⁸]PDGF-BB by using an enzyme immunoassay (Boehringer Mannheim Biochemica Glycan Detection Kit) (results not shown). Human PDGF-BB has no consensus sites for N-linked glycosylation, but recombinant PDGF-BB secreted from yeast using a similar

Table 3. N-Terminal amino acid sequences for purified PDGF-BB and mutants [Ser²⁸]PDGF-BB and [Pro³²]PDGF-BB

Values are derived from the pmol of amino acids detected, given as the mean for three or four batches (purified from separate cultures) of PDGF. The internal cleavage of PDGF at Thr-33 is given as a percentage of the total PDGF, independent of the small amount of processing at the N-terminus (Ser-1–Thr-6) that probably occurs in both the uncleaved and cleaved forms.

PDGF	Residue ...	N-Terminal processing (%)						Internal cleavage (%)
		Ser-1	Leu-2	Gly-3	Ser-4	Leu-5	Thr-6	Thr-33
PDGF-BB (<i>n</i> = 4)		69	4	22	5			49
[Ser ²⁸]PDGF-BB (<i>n</i> = 4)		90	4	4			2	10
[Pro ³²]PDGF-BB (<i>n</i> = 3)		86	6	4			4	0

alpha-factor secretion system has been shown to have O-linked glycosylation at six of nine threonine residues and at serine-26 (Settineri *et al.*, 1990).

After reduction of PDGF-BB, two bands of approx. *M_r* 14000 and 10000 are apparent (Fig. 6). N-Terminal sequencing of PDGF-BB shows that approx. 50 % of the molecules are cleaved after Arg-32 (Table 3). The calculated *M_r* for intact PDGF-BB (1–109) is 12300 and for the cleaved molecule (33–109) it is 8700.

Analysis of mutant proteins [Ser²⁸]PDGF-BB and [Pro³²]PDGF-BB by SDS/PAGE showed a single band both before and after reduction (Fig. 6). N-Terminal sequencing indicates a low level of cleavage (approx. 10 %) of [Ser²⁸]PDGF-BB at Arg-32 (Table 3). Replacement of Arg-32 with a proline residue prevents cleavage at this site, and no cleavage is detected at the potential KEX2 cleavage site (Arg-27–Arg-28) by N-terminal sequencing of mutant [Pro³²]PDGF-BB (Table 3).

c-sis (Amersham), which is used as a standard in the biological assays, was also analysed by SDS/PAGE. Under non-reducing conditions, two major bands are evident at *M_r* 24000 and *M_r* 34000 (Fig. 6). After reduction, several bands are detected at *M_r* 12000–18000. This heterogeneity is attributed to variable glycosylation and processing at the C-terminus during production of c-sis from Chinese-hamster ovary cells.

Biological analysis

The protein concentration of purified PDGF was determined, using the calculated absorption coefficient, from the *A*₂₈₀. The biological activity of purified PDGF-BB, [Ser²⁸]PDGF-BB or [Pro³²]PDGF-BB was calculated from a standard curve for a known quantity of c-sis. The protein level of c-sis is determined from the absorbance at 280 nm and amino acid analysis (Amersham). The ED₅₀ for c-sis in the mitogenic assay was 1.06 ± 0.08 ng/ml, and in the inositol-lipid-turnover assay it was 6.08 ± 0.83 ng/ml (Table 4).

Both mutants show biological activity in mitogenic and inositol-lipid-turnover assays (Table 4). Comparison of biological activity by titration of 100 µg/ml samples (determined by absorbance at 280 nm) shows significantly reduced (*P* < 0.001) potency for PDGF-BB in the mitogenic assay compared with the mutant proteins [Ser²⁸]PDGF-BB and [Pro³²]PDGF-BB (Table 4). The two mutants also stimulated inositol-lipid turnover to a greater extent than did PDGF-BB (*P* < 0.001), suggesting that the biological activity of PDGF-BB is impaired (Table 4). The ED₅₀ values for inositol-lipid turnover are higher than the ED₅₀ values in the mitogenic assay, which may be due to the difference in incubation time for PDGF in these assays (20 min and 22 h respectively) and/or the heterogeneous nature of the c-sis standard (Fig. 6).

An e.l.i.s.a. was used to quantify PDGF in yeast supernatant, but was shown to overestimate PDGF levels in samples with

Table 4. Biological activity of PDGF-BB and mutants [Ser²⁸]PDGF-BB and [Pro³²]PDGF-BB

The ED₅₀ values shown for the mitogenesis and inositol-lipid-turnover assays are the means ± s.d. for five samples from three batches of PDGF-BB, [Ser²⁸]PDGF-BB and [Pro³²]PDGF-BB in which the absolute protein concentration of each stock solution (100 µg/ml) was determined by *A*₂₈₀. The concentration of c-sis standard was initially determined by absorbance at 280 nm and amino acid analysis (Amersham), and the ED₅₀ values shown are means ± s.d. for five samples.

PDGF	ED ₅₀ (ng/ml)	
	Mitogenic activity	Inositol-lipid turnover
PDGF-BB	1.95 ± 0.19	7.38 ± 1.17
[Ser ²⁸]PDGF-BB	0.85 ± 0.07	3.19 ± 0.48
[Pro ³²]PDGF-BB	0.81 ± 0.08	2.42 ± 0.29
c-sis standard	1.06 ± 0.08	6.08 ± 0.83

initial concentrations of greater than 5 µg/ml (results not shown). The assay did not discriminate between PDGF-BB, [Ser²⁸]PDGF-BB and [Pro³²]PDGF-BB; samples of an initial concentration of 100 µg/ml (by absorbance at 280 nm) were estimated to be 263, 247 and 297 µg/ml respectively by e.l.i.s.a. It is not known whether the estimation of PDGF levels in yeast supernatant by mitogenic activity and e.l.i.s.a. yield an accurate measurement of specific activity, or whether active mutant proteins are selectively purified from inactive proteins or peptides.

DISCUSSION

To facilitate studies on the structure and function of PDGF-BB, we adopted a yeast expression strategy. The gene for PDGF-BB was truncated by removing the region coding for the basic C-terminal tail to produce a protein which corresponds to the mature form of the molecule (Johnsson *et al.*, 1984). Previous work has shown that deletion of PDGF-BB to Ala-103 does not affect the biological activity of the molecule (Hannink *et al.*, 1986; Sauer *et al.*, 1986). The minimal region of PDGF-BB required for activity is thought to be Cys-16 to Cys-97 (Sauer & Donoghue, 1988).

N-Terminal processing observed in PDGF-BB and mutants [Ser²⁸]PDGF-BB and [Pro³²]PDGF-BB up to Thr-6 (10–30 %) would not be expected to affect biological activity, since recombinant PDGF without the first 12 amino acids was shown to have mitogenic activity comparable with that human PDGF (Hoppe *et al.*, 1989). PDGF derived from human platelets also

shows substantial *N*-terminal processing to Thr-6 (Hart *et al.*, 1990). In addition to *N*-terminal processing, internal cleavage after Arg-32 is found in approx. 50 % of the wild-type PDGF-BB isolated from yeast. Human platelet-derived PDGF is also cleaved at Thr-33, in approximately the same proportion (54 % internal cleavage) as observed in PDGF-BB secreted from yeast (Hart *et al.*, 1990). Proteolytic processing at this site may be part of the normal regulation of PDGF *in vivo*.

In yeast, the product of the *KEX2* gene is required for the proteolytic processing of the alpha-factor-mating-pheromone and the killer-toxin precursors (Julius *et al.*, 1984). The product of the *KEX2* gene has been shown to have structural and functional similarity to the members of the subtilisin-like serine-proteinase family (Mizuno *et al.*, 1988). A human counterpart of the *KEX2* gene also exists in mammalian cells (Fuller *et al.*, 1989). The protein is a membrane-bound Ca^{2+} -dependent endopeptidase located in the Golgi and cleaves on the carboxy side of a pair of basic residues (Julius *et al.* 1984; Mizuno *et al.*, 1989). It is not clear whether the *KEX2* product is responsible for the cleavage at Thr-33 in yeast, as this site does not contain a pair of basic residues. However, *KEX2* exhibits cleavage at additional sites, such as proline-arginine (Mizuno *et al.*, 1989). The mutation in [Ser²⁸]PDGF-BB disrupts a potential *KEX2* site in PDGF-BB at Arg-27-Arg-28, but also reduces cleavage after arginine-32 to 10 %. It is possible that there may be an initial *KEX2* cleavage after Arg-28, which then renders the Arg-32 site susceptible to a second cleavage. However, if this were the case, the mutation in [Pro³²]PDGF-BB, which abolishes cleavage after position 32, would reveal the initial cleavage after position 28. This was not found, and it seems more likely, therefore, that [Ser²⁸]PDGF-BB reduces accessibility of a proteinase to the cleavage site at Arg-32. Since [Ser²⁸]PDGF-BB and [Pro³²]PDGF-BB molecules have similar secondary structures to the wild-type molecule (see the accompanying paper by Craig *et al.*, 1991), a gross structural change does not appear to be responsible for the altered cleavage specificity.

The biological activity of recombinant PDGF-BB in yeast is clearly impaired compared with the uncleaved mutant proteins (Table 4). This is not surprising in view of the fact that the region Ile-25-Phe-37, which encompasses the major cleavage sites, has been implicated in receptor binding (Giese *et al.*, 1990). It is possible that cleavage of a single chain might be sufficient to inactivate the PDGF homodimer. Activation of the receptor is known to require dimerization, presumably induced by its interaction with dimeric PDGF (Heldin *et al.*, 1989). PDGF-BB, cleaved in one or both of its subunits, may be unable to cross-link the receptor and provoke a mitogenic or inositol-lipid-turnover response. An alternative possibility, however, is that cleavage of one or both B-chains in this region may diminish the ability of PDGF to bind to its receptor and/or induce a mitogenic or inositol-lipid-turnover response. Our results indicate that approx. 50 % of the B-chains are cleaved in yeast-derived PDGF-BB. Assuming that cleavage of the dimer is a stochastic phenomenon, unaffected by prior cleavage in the other subunit and that cleavage does not affect PDGF stability, one might predict that only 25 % of the PDGF-BB molecules would be uncleaved and fully active. Consistent with this, the uncleaved mutant proteins [Ser²⁸]PDGF-BB and [Pro³²]PDGF-BB are some 2–3 times more potent than PDGF-BB in both mitogenesis and inositol-lipid-turnover assays. We propose a model in which heterodimeric (cleaved/uncleaved) PDGF-BB binds with lower affinity than uncleaved PDGF-BB. This model is testable by a combination of detailed receptor-binding studies and purification of the cleaved forms of PDGF-BB.

Site-directed mutagenesis, as described here, provides a method for production of uncleaved PDGF-BB, which can be used for

biological studies or as a 'background' molecule for other mutations. This method also provides an efficient expression and purification process for production of recombinant PDGF-BB from yeast.

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