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<sub>r</sub> 28000–31000 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<sup>onc</sup>, 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., 1988a).

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* (Hopp et al., 1990). Both A- and B-chains undergo proteolytic processing during expression and secretion in *E. coli*, 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<sub>2</sub>PO<sub>4</sub>/1.5 mM-KH<sub>2</sub>PO<sub>4</sub>/0.137 mM-NaCl/0.003 mM-KCl, pH 7.3); PDGF, platelet-derived growth factor; ED<sub>50</sub> concentration at which 50% of maximal stimulation is obtained.
**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 prcl1-407 prb1-1122 pep4-3 leu2 trpl 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 at 1% (w/v) glucose/synthetic complete medium without leucine to an *A*₅₆₀ 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 μl 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 (unduced) 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 chromatography on a 200 ml CM-Sepharose column (Pharmacia; 5 cm x 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 x 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, assayed 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 x 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 liter·g⁻¹·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 μg per track) were separated on 8–25% Phast gels (Pharmacia) for 75–90 V·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 P-40/PBS.
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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, [Ser19]PDGF-BB or [Pro30]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 x 10^4 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, [Ser19]PDGF-BB and [Pro30]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 x 10^4 per well. After 3 days, the cells were 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 Kyh (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.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 mM-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

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

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 [Ser19]PDGF-BB and [Pro30]PDGF-BB mutations are indicated. The restriction sites used to clone PDGF-B into pSW6 are shown.

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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 yeast Saccharomyces cerevisiae (Bennetzen & Hall, 1982), and a number of useful restriction sites engineered into the sequence. The sequence codes for a 160-aminoc 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 gene 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-B (Table 1). The presence of full-length and truncated PDGF-B in culture medium was identified by Western blot. Two species of M, 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-B, 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-B.

PDGF-B 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-B was being limited, the plasmid copy number, transcript levels and the intra- and extracellular concentrations of PDGF-B 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-B
Proteinase-resistant mutants of platelet-derived growth factor-BB

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). Mr standards (Bio-Rad prestained SDS/PAGE standards, low range) are in lane 8. Full-length PDGF-BB (1–160) is detected at Mr 42,000 and truncated PDGF-BB (1–109) at Mr 32,000.

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, Mr 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 Mr 32,000.

Table 2. Purification of PDGF-BB from yeast supernatant

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Volume (ml)</th>
<th>Mitogenic activity (µg/ml)</th>
<th>PDGF (mg)</th>
<th>Recovery (%)</th>
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</thead>
<tbody>
<tr>
<td>Yeast supernatant</td>
<td>5500</td>
<td>0.62</td>
<td>3.41</td>
<td>100</td>
</tr>
<tr>
<td>Concentrated supernatant</td>
<td>750</td>
<td>3.88</td>
<td>2.91</td>
<td>85</td>
</tr>
<tr>
<td>CM-Sepharose and</td>
<td>55</td>
<td>39.82</td>
<td>2.19</td>
<td>64</td>
</tr>
<tr>
<td>phenyl-Sepharose 2 x Sephadex G-25M</td>
<td>70</td>
<td>16.28</td>
<td>1.14</td>
<td>33</td>
</tr>
</tbody>
</table>

were detected 1 day after induction at approx. 0.6 µg/ml and remained relatively high over 6 days. However, it took 6 days for significant quantities of PDGF-BB to accumulate in the medium (0.3 µg/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 [Ser<sup>45</sup>PDGF-BB disrupts one of the preferred cleavage sites (Arg–Arg) of the yeast KEX2 protease (Julius et al., 1984). The second mutant, [Pro<sup>49</sup>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 [Ser<sup>45</sup>PDGF-BB and [Pro<sup>49</sup>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 (0.4–1.5 µg/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-
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- Mr 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 mM-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 [Ser30]PDGF-BB and [Pro32]PDGF-BB were purified to > 95% by 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 Mr 28000 by SDS/PAGE under non-reducing conditions. The calculated Mr 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. Mr 26000 (results not shown), and carbohydrate can be detected both in PDGF-BB and in [Ser30]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 conditions.
Table 3. N-Terminal amino acid sequences for purified PDGF-BB and mutants [Ser<sup>32</sup>IPDGF-BB and [Pro<sup>32</sup>IPDGF-BB

<table>
<thead>
<tr>
<th>PDGF</th>
<th>Residue...</th>
<th>N-Terminal processing (%)</th>
<th>Internal cleavage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ser-1</td>
<td>Leu-2 Gly-3 Ser-4 Leu-5</td>
<td>Thr-6 Thr-33</td>
</tr>
<tr>
<td>PDGF-BB (n = 4)</td>
<td>69 4 22 5</td>
<td>2 49</td>
<td></td>
</tr>
<tr>
<td>[Ser&lt;sup&gt;32&lt;/sup&gt;IPDGF-BB (n = 4)</td>
<td>90 4 4 0</td>
<td>10 0</td>
<td></td>
</tr>
<tr>
<td>[Pro&lt;sup&gt;32&lt;/sup&gt;IPDGF-BB (n = 3)</td>
<td>86 6 4 0</td>
<td>0 0</td>
<td></td>
</tr>
</tbody>
</table>

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<sub>r</sub> 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<sub>r</sub> for intact PDGF-BB (1-109) is 12300 and for the cleaved molecule (33-109) it is 8700.

Analysis of mutant proteins [Ser<sup>32</sup>IPDGF-BB and [Pro<sup>32</sup>IPDGF-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 [Ser<sup>32</sup>IPDGF-BB at Arg-32 (Table 3). Replacement of Arg-32 by 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 [Pro<sup>32</sup>IPDGF-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<sub>r</sub> 24000 and M<sub>r</sub> 34000 (Fig. 6). After reduction, several bands are detected at M<sub>r</sub> 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<sub>280</sub>. The biological activity of purified PDGF-BB, [Ser<sup>32</sup>IPDGF-BB or [Pro<sup>32</sup>IPDGF-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 ED<sub>50</sub> 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 < 0.0001) potency for PDGF-BB in the mitogen assay compared with the mutant proteins [Ser<sup>32</sup>IPDGF-BB and [Pro<sup>32</sup>IPDGF-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 ED<sub>50</sub> values for inositol-lipid turnover are higher than the ED<sub>50</sub> 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 initial concentrations of greater than 5 µg/ml (results not shown).

The assay did not discriminate between PDGF-BB, [Ser<sup>32</sup>IPDGF-BB and [Pro<sup>32</sup>IPDGF-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 [Ser<sup>32</sup>IPDGF-BB and [Pro<sup>32</sup>IPDGF-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 Ca2+-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 mitogenetic 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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REFERENCES


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