Characterization and *N*-terminal sequence of human platelet proteoglycan

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Human platelet proteoglycan (P.PG) was prepared from a 4 M-guanidinium chloride platelet extract in the presence of proteinase inhibitors. The purification procedure included CsCl-density-gradient centrifugation, DEAE-Sepharose CL-6B ion-exchange chromatography and f.p.l.c. on a Mono Q HR 5/5 column. P.PG was recovered as a polydisperse molecule, but the protein core appeared to be at least 90 % homogeneous. This observation could be due to partial proteolysis of the core protein during extraction. The *N*-terminal sequence of the human P.PG core protein was determined up to residue 66 and was shown to be highly homologous to the propeptide of an embryonic rat yolk-sac tumour proteoglycan (PG19); the significance of this homology is discussed.

INTRODUCTION

Proteoglycans are defined as macromolecules containing at least one glycosaminoglycan chain covalently bound to a core protein. They have been identified in a broad spectrum of tissues and have been proposed to participate in a multitude of functions in the cells and tissues as a result of their polymorphism (for reviews see refs. [1] and [2]). Glycosaminoglycans have long been known to be present in platelets, and their distribution mainly in α -granules as well as on surface membranes has been demonstrated [3,4]. An interaction between platelet proteoglycan (P.PG) and platelet factor 4 has been demonstrated, and it has been postulated that one possible role of P.PG is the 'packaging' of platelet factor 4 in platelet α -granules [5]. The P.PG might therefore be a platelet factor 4 carrier for delivery to sites of blood-vessel injury. An inhibitor of complement subcomponent C1q has been identified in human serum as to be proteoglycan [6]. On the basis of the nature and the size of their glycosaminoglycan side chains, the proteoglycans involved in these two functions might be related despite the fact that average molecular masses appeared to be somewhat different; this observation could be the result of partial degradation during the preparations. P.PG has recently been obtained from freshly prepared human platelets and partially characterized [7] as a 136 kDa molecule containing four chondroitin 4-sulphate chains, each with an apparent molecular mass of 28 kDa. This P.PG was very similar to the C1q inhibitor previously characterized. It is therefore likely that P.PG acts as a C1q inhibitor in addition to being a carrier of platelet factor 4 [5–7]. Despite the importance of the two above-mentioned biological roles, a molecular study of P.PG protein core is still lacking. The determination of the protein structure of P.PG seemed to us to be a prerequisite in the elucidation of its possible functions.

EXPERIMENTAL

Materials

All the chromatographic media, namely DEAE-Sepharose CL-6B, Superose 6 (HR/10/30 column) and Mono Q (HR 5/5 column), were from Pharmacia Biotechnology (Uppsala, Sweden); the two last-mentioned media were fitted to the Pharmacia f.p.l.c. apparatus and the elution profile recordings were obtained with the aid of a Pharmacia UV-1 monitor at 280 nm. The Biotrap apparatus was from Schleicher und Schüll (Dassel, Germany). Chondroitinase AC (chondroitin AC lyase; EC 4.2.2.5) and chondroitinase ABC (chondroitin ABC lyase, EC 4.2.2.4) were from Sigma Chemical Co. (St Louis, MO, U.S.A.). All other reagents (analytical grade) were from Prolabo (Paris, France), Merck (Darmstadt, Germany), Carlo Erba (Milan, Italy) or Sigma Chemical Co. The reagents used for protein sequencing experiments were from Applied Biosystems (Foster City, CA, U.S.A.).

Purification of the platelet proteoglycan

A typical platelet proteoglycan (P.PG) preparation was carried out with fresh concentrated platelet-rich human plasma from 20 random donors, regardless of their blood type, provided by the Centre de Transfusion Sanguine Hospitalier Lariboisière, Paris, France. Contaminating leucocytes and erythrocytes were discarded by pelleting during a 10 min centrifugation at 200 *g*; the platelets in the supernatant were then submitted to a 10 min centrifugation at 1500 *g* at 20 °C. The platelet pellet was washed three times with 100 ml of 0.9 % NaCl/0.33 % EDTA before extraction of P.PG. This was carried out for 3 h at 4 °C in 100 ml of 4 M-guanidinium chloride/50 mM-sodium acetate buffer, pH 5.8, containing 5 mM-benzamidine, 10 mM-6-amino-hexanoic acid and 10 mM-EDTA as proteinase inhibitors. Solid CsCl was added to the extract up to a density of 1.4 g/ml and the mixture was submitted to density-

Abbreviations used: P.PG, platelet proteoglycan; PG19, rat yolk-sac tumour proteoglycan.

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gradient centrifugation (35000 rev./min in a Beckman L8-55 ultracentrifuge, 60 Ti rotor, for 40 h at 20 °C); the P.PG-containing fraction was recovered in the bottom quarter of the centrifuge tube. It was exhaustively dialysed against 7.6 M-urea/50 mM-Tris/HCl buffer, pH 7.6 (buffer A), at 4 °C. This dialysis method was less efficient in desalting when compared with the classical dialysis method against water, but allowed the material to be maintained in a soluble state. The dialysed P.PG fraction was directly applied to a DEAE-Sephacrose CL-6B column (12 cm × 1.5 cm) equilibrated in buffer A. The column was successively washed with buffer A (30 ml), then with buffer A containing 0.2 M-NaCl (50 ml), and the elution of P.PG was achieved with buffer A containing 1 M-NaCl (flow rate 20 ml/h). The 1 M-NaCl-eluted fraction was made 0.2 M with respect to NaCl by a 5-fold dilution with buffer A and was directly applied to a Mono Q HR 5/5 column equilibrated in buffer A containing 0.2 M-NaCl. Elution was performed with a linear 95 ml NaCl gradient (0.2–1 M) in buffer A (flow rate 0.5 ml/min); the P.PG-containing fractions were exhaustively dialysed against distilled water at 4 °C and dried under vacuum.

Characterization of the platelet proteoglycan

Superose 6 gel filtrations of P.PG were carried out with 0.15 M-NaCl/50 mM-Tris/HCl buffer, pH 7.6 (buffer B) as eluent, whereas 4 M-guanidinium chloride/50 mM-sodium acetate buffer, pH 5.8 (buffer C), was used for the gel filtration of the chondroitinase-treated P.PG (P.PG core) on the same chromatographic medium (HR 10/30 column, flow rate 0.5 ml/min).

Polyacrylamide/agarose-gel electrophoresis was performed in accordance with McDewitt & Muir [8] on 2.4% polyacrylamide/1.2% agarose gels. SDS/polyacrylamide-gel electrophoresis was performed by the procedure of Laemmli [9] on 15% polyacrylamide gels. Glycosaminoglycans were stained with 0.1% Toluidine Blue in 1% acetic acid, and proteins with 0.05% Coomassie Brilliant Blue G in methanol/acetic acid/water (5:1:5, by vol.).

The electroelution of the P.PG core was achieved in accordance with Jacobs & Clad [10] with the Biotrap apparatus with 0.1% SDS/25 mM-Tris/HCl/192 mM-glycine electrophoresis buffer, pH 8.3 (8 h at 200 V). The electroeluted materials were dialysed against distilled water, dried under vacuum and solubilized in 100 µl of 4 M-guanidinium chloride before precipitation for 24 h at -20 °C by addition of 5 vol. of ethanol. The resulting pellets were washed three times with cold 70% (v/v) ethanol before sequencing experiments. *N*-Terminal amino acid sequences were determined with an Applied Biosystems 470A sequencer equipped with a model 120 phenylthiohydantoin analyser. Amino acid analyses were performed after total acid hydrolysis (6 M-HCl at 110 °C in the presence of 0.2% 2-mercaptoethanol for 18 h under vacuum) with the aid of a Biotronik Autoanalyzer (model LC 6000).

Chondroitinase treatments were carried out at 37 °C for 4 h in buffer B with 0.0001 enzyme unit/µg of hexuronate for chondroitinase AC as well as for chondroitinase ABC. CNBr treatment was performed in 70% formic acid at 20 °C for 18 h as previously described [11]. Uronates were determined by the carbazole method [12]. Deglycosylation in HF/pyridine was carried out for 4 h at 20 °C under N₂ [13].

RESULTS

Purification of the platelet proteoglycan

The platelets were completely solubilized in 4 M-guanidinium chloride/50 mM-sodium acetate buffer, pH 5.8, containing proteinase inhibitors. Upon CsCl addition up to a density of 1.4 g/ml the extract became cloudy and a substantial gel formed at the top of the centrifuge tube during the density-gradient centrifugation. About 80% of the hexuronate was recovered in the bottom quarter of the tube after this step. After extensive dialysis against buffer A at 4 °C no precipitation occurred, and the material was directly applied to a DEAE-Sephacrose CL-6B column equilibrated in the same buffer. The elution was achieved by a two-step procedure (Fig. 1); approx. 10% of the hexuronate was eluted in the 0.2 M-NaCl fraction and 40% was recovered in the 1 M-NaCl fraction. About 50% of the applied hexuronates was not bound to the column; this observation might be due to partial inhibition of binding to the DEAE-Sephadex column as a result of the dialysis conditions (see the Experiment section). Approx. 70–75% of the hexuronates was bound to the Mono Q HR 5/5 column; this material was eluted between 0.70 M- and 0.85 M-NaCl as four poorly resolved peaks (I, II, III and IV in Fig. 2). After extensive dialysis against distilled water each individual fraction was dried under vacuum. Scheme 1 summarizes the fractionation procedure.

Characterization of the platelet proteoglycan polydispersity in fractions I, II, III and IV (Fig. 2)

Superose 6 HR 10/30 gel filtration in buffer B of P.PG fractions I, II, III and IV recovered after Mono Q HR 5/5 ion-exchange chromatography is illustrated in Fig. 3. Each fraction was eluted as a single hexuronate-

Fig. 1. DEAE-Sephacrose CL-6B chromatography of the P.PG-enriched fraction recovered after CsCl-density-gradient centrifugation

The column (12 cm × 1.5 cm) was equilibrated with buffer A (see the Experimental section) and eluted stepwise with 50 ml of 0.2 M-NaCl and then with 50 ml of 1 M-NaCl in buffer A; the flow rate was 20 ml/h, and 2 ml fractions were collected. —, A_{280} ; ----, hexuronate. The 1 M-NaCl fraction indicated by the bar was pooled as the P.PG fraction.

Fig. 2. Ion-exchange chromatography of P.PG on a Mono Q column

The 1 M-NaCl fraction recovered from DEAE-Sephacel CL-6B (Fig. 1) was applied to the Mono Q HR 5/5 column after a 5-fold dilution in buffer A. The f.p.l.c. was run as indicated in the Experimental section; 0.5 ml fractions were collected. ·····, NaCl gradient (0.2–1 M); —, A_{280} ; ----, hexuronate. Four hexuronate peaks, I, II, III and IV, were pooled as P.PG fractions.

Scheme 1. Purification of human platelet proteoglycan

For full details see the Experimental section.

containing peak coinciding with a 280 nm-absorbing peak; the hexuronate recoveries ranged between 95 and 98% for the different P.PG fractions during this step. The partition coefficients K_{av} were 0.30, 0.27, 0.24 and 0.22 for fractions I, II, III and IV respectively. The hexuronate/ A_{280} ratio increased from fraction I to fraction IV: it was 3.6-fold higher for fraction IV than for fraction I. On polyacrylamide/agarose-gel electrophoresis the mobility decreased from fraction I to fraction IV (Fig. 4a).

These observations suggest that P.PG is polydisperse; the polydispersity is reflected by the retention on the Mono Q HR 5/5 column, indicating a charge heterogeneity. The latter is accompanied by a shift in the

Fig. 3. Superose 6 gel filtration of P.PG fractions I, II, III and IV

The fractions isolated after ion-exchange chromatography on the Mono Q column (Fig. 2) were applied in 200 μl of buffer B on the HR 10/30 column; the flow rate was 0.5 ml/min. The retarded u.v.-absorbing peaks presumably represent impurities or artifacts of the chromatography monitoring system. —, A_{280} ; ----, hexuronate.

P.PG K_{av} values. The more acidic the P.PG, the higher its hexuronate/protein ratio and the larger its size. Therefore we attributed, in a first approximation, this heterogeneity to a variation in the glycosaminoglycan content of the P.PG.

Fig. 4. Electrophoretic characterization of the P.PG fractions I, II, III and IV isolated in Fig. 2

(a) Polyacrylamide/agarose gel electrophoresis. Samples (1.25 μ g of hexuronate) of the P.PG fractions I, II, III and IV were applied. After electrophoresis the gel was stained with Toluidine Blue. (b) SDS/polyacrylamide-gel electrophoresis of chondroitinase ABC-treated P.PG fractions. Samples (12.5, 17.5, 40 and 30 μ g of hexuronate respectively) of P.PG fractions I, II, III and IV were applied. After electrophoresis the gel was stained with Coomassie Blue. Note the presence of a faint band (band X) in addition to the common major one.

The identity of the P.PG protein core in fractions I, II, III and IV was demonstrated after chondroitinase ABC digestion. A major gel-penetrating band common to all the four digested samples was characterized on SDS/polyacrylamide-gel electrophoresis by Coomassie Blue staining (Fig. 4b); non-digested samples did not give rise to any gel-penetrating band under these conditions. No material could be detected above the major band in any sample when the gels were stained with Coomassie Blue or Toluidine Blue. An additional faint and diffuse band (band X in Fig. 4b) could, however, be detected by Coomassie Blue staining, just below the major one, mainly in the digests of fractions III and IV. This result indicates that the protein cores of P.PG in fractions I, II, III and IV have an identical size, but the minor component (band X) might represent a second core protein, as discussed below. Therefore the subsequent experiments dealing with P.PG molecular characterization were performed on pooled fractions I, II, III and IV further purified on Superose 6 in order to remove any low-molecular-mass 280 nm-absorbing material (Fig. 6a).

Molecular characterization of platelet proteoglycan

Determination of the protein core size of P.PG. After chondroitinase AC or ABC treatment the apparent molecular mass of the P.PG core protein was estimated by SDS/polyacrylamide-gel electrophoresis (Fig. 5a). It was estimated at 27 kDa after chondroitinase ABC treatment, whereas it was 24 kDa after chondroitinase AC digestion; in both cases a faint diffuse band was observed below the major one. A sample treated with a mixture of the two chondroitinases gave rise to the 24 kDa band. After HF/pyridine treatment the P.PG core was characterized as a major 20 kDa compound; a minor band was also detected as a smear with an approximate apparent molecular mass of 17 kDa (Fig. 5b). When HF/pyridine treatment was performed on a previously chondroitinase ABC-treated P.PG the same major band (20 kDa) was still detected. These results suggest that the P.PG core apparent molecular mass does not exceed 20 kDa; the existence of a compound of a slightly smaller size is indicated as a smear.

N-Terminal sequence of P.PG. The purification of the P.PG protein core obtained after chondroitinase ABC treatment was achieved by Superose 6 gel filtration with buffer C as eluent. Under these conditions the P.PG protein core was eluted as a symmetrical peak with a K_{av} value of 0.60, whereas non-treated P.PG was eluted with a K_{av} of 0.21 (Fig. 6). The totality of the 280 nm-absorbing material was recovered in the 0.60 K_{av} peak when compared to the non-treated P.PG. The amino

Fig. 5. Estimation of the molecular mass of the P.PG core protein

(a) P.PG treated with chondroitinases AC, ABC and AC + ABC. The 67 kDa band corresponds to the bovine serum albumin present in the chondroitinase AC. (b) HF/pyridine-treated P.PG (HF). Standard markers (lane O) were lysozyme (14.4 kDa), soya-bean trypsin inhibitor (20 kDa), carbonic anhydrase (30 kDa), ovalbumin (43 kDa), bovine serum albumin (67 kDa) and phosphorylase b (94 kDa).

Fig. 6. Purification of the 27 kDa P.P.G. core protein obtained after chondroitinase ABC treatment

Superose 6 gel filtration was carried out with buffer C as eluent. (a) Untreated P.P.G.; (b) chondroitinase ABC-treated P.P.G.; (c) CNBr-treated P.P.G. The 27 kDa protein core and the CN1 peptide were recovered in the peaks indicated by the bars in (b) and (c) respectively. See the comment in the legend to Fig. 3 regarding the retarded peaks; in panels (b) and (c) these may also represent degradation products.

acid compositions of P.P.G. and of its Superose 6-purified core are presented in Table 1; glycine, serine, aspartic acid and glutamic acid were predominant amino acids.

The Superose ABC core (chondroitinase ABC-treated P.P.G., purified by Superose 6 gel filtration, K_{av} 0.60) was

Table 1. Amino acid composition of the human platelet proteoglycan (P.P.G.)

P.P.G. was purified as indicated in the Experimental section. P.P.G. core was obtained after chondroitinase ABC digestion and purified by Superose 6 gel filtration. No correction was made for losses during hydrolysis. Abbreviation: N.D., not determined.

	Amino acid composition (residues/1000 residues)	
	P.P.G.	P.P.G. core protein
Asp	153	149
Thr	28	26
Ser	149	135
Glu	138	138
Pro	54	55
Gly	126	125
Ala	28	24
Cys	N.D.	N.D.
Val	16	17
Met	16	16
Ile	22	22
Leu	107	106
Tyr	26	31
Phe	47	67
Lys	19	21
His	14	14
Arg	57	54

submitted to automated Edman degradation. Two *N*-terminal sequences were characterized: one of them, representing 10% of the material, was DLFPK..., and the other (90%) was YPTQR.... When non-chondroitinase treated P.P.G. was submitted to the same experiment, both sequences were again characterized. The following experiment was carried out to clarify the situation.

Chondroitinase ABC-treated P.P.G. was submitted to SDS/polyacrylamide-gel electrophoresis, and the 27 kDa band and the diffuse smear below were excised without staining and electroeluted. After dialysis against distilled water the materials were solubilized in 4 M-guanidinium chloride and precipitated with cold ethanol. The 27 kDa band was homogeneous upon sequencing, and its *N*-terminal amino acid sequence was determined up to residue 41 (Fig. 7). The *N*-terminal amino acid sequence of the material excised in the smear below the 27 kDa band (band X in Fig. 4b) was determined up to residue 21 (Fig. 7).

After CNBr treatment of P.P.G., one major peak (CN1) was isolated by Superose 6 gel filtration in buffer C with a K_a value identical with that of native P.P.G. (Fig. 6c). The *N*-terminal amino acid sequence of this fragment was determined up to residue 24 (Fig. 7). This peptide overlaps the two previously characterized *N*-terminal sequences in the P.P.G. preparations, demonstrating that the DLFPK... sequence begins in fact at position 46 in the 27 kDa protein core of P.P.G.

DISCUSSION

Platelet proteoglycan was isolated in a degree of purity allowing the characterization of the structure of its

Fig. 7. *N*-Terminal sequence of human platelet proteoglycan (P.PG), and comparison with rat yolk-sac tumour proteoglycan (PG19) [14]

→, Sequence characterized on chondroitinase ABC-treated P.PG (27 kDa band) by automated Edman degradation; CN1 is the CNBr fragment isolated in Fig. 6(c); band X was isolated as indicated in the text. Numbers refer to amino acid positions in P.PG. Identical amino acids between rat PG19 and P.PG are boxed. -, Deletion (introduced in rat PG19 for optimal sequence alignment). X, As yet undetermined amino acid. ↓, Propeptide processing site in rat PG19.

protein core. After a short-term extraction (3 h), 10% of the core appeared as an *N*-terminal-shortened molecule when compared with the major *N*-terminal-sequence-characterized protein after chondroitinase ABC treatment. This observation indicated either that P.PG protein core exists *in vivo* as two entities or that proteolysis occurred during the preparation. We have observed that long-term extraction (24 h) resulted in an increase of the *N*-terminal-shortened molecule up to 60% of the total core (J.-P. Périn, F. Bonnet, P. Maillet & P. Jollès, unpublished work), indicating that proteolysis occurs during the extraction step.

P.PG was characterized as containing polydisperse molecules, the polydispersity being due to variations in the degrees of glycosylation and presumably sulphatation. Silvestri *et al.* [6] reported the polydispersity of a chondroitin 4-sulphate platelet proteoglycan with molecular masses ranging from 45 to 750 kDa (average value 175 kDa) containing 9% protein. Okayama *et al.* [7] characterized a platelet proteoglycan (molecular mass 136 kDa), presumably identical with the former, with four chondroitin 4-sulphate side chains and containing 15% protein. We estimated the molecular mass of the P.PG protein core to be 20–27 kDa depending on the deglycosylation method used before SDS/polyacrylamide-gel-electrophoretic analysis; this value fits rather well with calculated values for the molecular mass of the core from data of Silvestri *et al.* [6] and Okayama *et al.* [7] (16–20 kDa). On the other hand the amino acid composition of the isolated P.PG in the present work is in good agreement with the data of Okayama *et al.* [7], suggesting that the P.PG that we have isolated is identical with their preparation.

The *N*-terminal amino acid sequence of P.PG was deduced from the three overlapping sets of sequence data presented in the Results section (the intact P.PG, the CN1 fragment and band X). When compared with previously established proteoglycan core structures, a very high degree of homology was found between P.PG and a chondroitin sulphate proteoglycan (PG19) characterized by Bourdon *et al.* [14] from rat yolk-sac tumour cells. It has been postulated that PG19 core protein is translated as a preprotein, and the putative

signal peptide cleavage site was localized just before a YPARRAR... sequence at position 27 in the PG 19 preprocore. The P.PG *N*-terminal sequence can be aligned with the PG19 proprotein core and covers the total length of the PG19 propeptide and a part of the processed PG19 molecule (Fig. 7). We have indicated that during its extraction P.PG is shortened in its *N*-terminal region, the first 45 amino acid residues being split off at a position very close to the cleavage site of the propeptide in PG19. This observation raises the possibility that the first 45 amino acid residues in P.PG in fact consist of a core propeptide that is partially removed after lysis of the platelets. We possess evidence that a Ser-Gly repeated structure exists in P.PG as in PG19, its length being apparently shorter in the former (J.-P. Périn, F. Bonnet, P. Maillet & P. Jollès, unpublished work).

In conclusion, in human platelets a chondroitin sulphate proteoglycan has been characterized whose protein core is of relatively small size; it could be a representative of a widespread class of the proteoglycan family that could be synthesized as a pre-form before exposure to the extracellular medium.

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