Annexin proteins PP4 and PP4-X

Comparative characterization of biological activities of placental and recombinant proteins

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The human placental proteins PP4 and PP4-X, belonging to the annexin protein family, were expressed in *Escherichia coli* at high yield. The proteins were purified to homogeneity. The physicochemical parameters of the recombinant proteins were determined and compared with those of their natural placental counterparts. Except for a minor change in the pI, the proteins appeared to be indistinguishable by several criteria. Both recombinant PP4 and recombinant PP4-X were biologically active in a thromboplastin inhibition test and in a phospholipase A₂ inhibition test.

INTRODUCTION

Since the assumption that the major action of glucocorticoids is associated with the induction of the phospholipase A₂ inhibitory proteins macrocortin or lipomodulin was first made [1,2], a whole class of membrane binding proteins was discovered which could possibly mediate this action [3]. This protein family was called 'lipocortins', 'calpactins' or 'annexins', based on their property of binding both to phospholipid membranes and to actin filaments in a calcium-dependent manner.

Under normal conditions cellular arachidonic acid is covalently bound to complex lipids such as phospholipids and triacylglycerols. The release of arachidonic acid is largely influenced by the action of phospholipases. In particular, hydrolysis by phospholipase A₂ represents the first step in the formation of eicosanoids, i.e. hydroxyeicosatetraenoic acids, prostaglandins and leukotrienes, with their distinct and often adverse biological effects in inflammation, thrombosis and other pathological events. The synthesis of annexins represents intracellular potential for protection of membrane lipids from degradation by phospholipases and subsequent liberation of arachidonic acid.

Annexin proteins are able to interact with reactive surfaces in the form of negatively charged phospholipids, which are commonly exposed during initiation of blood coagulation [4]. As a result, blood clotting factors are hindered from associating with membrane-bound enzymes and cofactors. By this mode of action, activation of prothrombin and the subsequent formation of fibrin clots is prevented.

Annexins have been detected in different species, organs and cell types [3,5]. Placenta protein 4 (PP4) was first isolated from human placenta by Bohn *et al.* [6]. The cloning and sequencing of its cDNA [7] revealed sequence similarity with known primary structures of other annexins. Other investigators have reported the expression in *Escherichia coli* of cDNA coding for an anticoagulant protein, which in the original communication was named 'endonexin II' [8], 'vascular anticoagulant' (VAC) [9] or 'inhibitor of blood coagulation' [10]. Subsequently it became clear that all of these cDNAs encode the same protein, which in fact is identical to PP4.

During the course of screening placental cDNA libraries for PP4, another annexin was detected, and named PP4-X [11]. The deduced amino acid sequence of PP4-X is identical with that of placenta anticoagulant protein II (PAP II), whose isolation from human placenta was first described by Tait *et al.* [12]. Recently the proteins PP4 and PP4-X were also named annexins V and IV respectively [13].

This paper reports on the high-level expression of PP4 and PP4-X in *E. coli* and on the isolation and characterization of the recombinant proteins, which we term rPP4 and rPP4-X. Comparison of the placental proteins with their recombinant counterparts indicated physicochemical, immunological and functional identity.

MATERIALS AND METHODS

Materials

Restriction enzymes, ligase and DNA polymerases were purchased from New England Biolabs and from Boehringer Mannheim, and were used according to the manufacturers' instructions. Isotopically labelled compounds were from NEN, pig phospholipase A₂ and prostaglandin F_{2α} (PGF_{2α}) from Sigma and 15-hydroxyeicosatetraenoic acid (15-HETE) from Paesel (Frankfurt, Germany). Culture medium PM 16 was obtained from Serva (Heidelberg, Germany), BW 755c from Hoechst AG (Frankfurt, Germany), the Supelclean LC-NH₂ column from Supelco (Bad Homburg, Germany) and the Nucleosil C18 column from Bischoff (Leonberg, Germany). DEAE-Sepharose and heparin-Sepharose, a chromatofocusing Mono P HR 5/20 column, Polybuffer and Superose TM 12 HR 10/30 were obtained from Pharmacia (Uppsala, Sweden). The bicinchoninic protein assay was from Pierce Chemical Co. All reagents used for coagulation tests were from Behringwerke AG (Marburg, Germany).

Construction of rPP4 and rPP4-X expression vectors

The cloning from human placental gene libraries and the characterization of cDNA coding for PP4 and PP4-X has been described previously [8,12]. For expression of rPP4 in *E. coli*, expression vector pTrc99A was employed, the construction of

Abbreviations used: (r)PP4, (recombinant) placenta protein 4; (r)PP4-X, (recombinant) placenta protein 4-X; (r)VAC, (recombinant) vascular anticoagulant; PAP I and II, placenta anticoagulant proteins I and II; ODTA, octadecatetraenoic acid; HETE, hydroxyeicosatetraenoic acid; PGF, prostaglandin F; TXA₂, thromboxane A₂; BMMC, 4-bromomethyl-7-methoxycoumarin; MMC esters, methylmethoxycoumarin esters; IPTG, isopropyl β-D-thiogalactopyranoside; BW 755c, 3-amino-1-[m-(trifluoromethyl)-phenyl]-2-pyrazoline].

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which has also been described previously [14]. In the pTrc vectors transcription of cloned genes is from the *trc* promoter [15], which is a more efficient derivative of *lac* and *trp* promoter sequences. Expression is under the control of the *lac* repressor and can be induced by addition of isopropyl β -D-thiogalactopyranoside (IPTG). The construction of pTrc99A-PP4 (5425 bp) has been described [14]. For expression of rPP4-X, pTrc99A was digested with *EcoRI* and the DNA was treated with mung bean nuclease. This gave the sequence 5' AACAGACCATGG 3', which was fused in frame by blunt-end ligation to the PP4-X cDNA located on a 1165 bp *BalI/HindIII* fragment. This latter fragment encodes the complete PP4-X sequence except for the ATG start codon, which was provided by the expression vector (underlined). This manipulation resulted in expression vector pTrc99A-PP4-X (5290 bp), whose correct sequence surrounding the ATG start codon was confirmed by DNA sequencing. The pTrc99A-PP4 and the pTrc99A-PP4-X vectors direct the expression of the unfused rPP4 and rPP4-X proteins respectively in *E. coli*. Isolation of plasmid DNA, preparation of DNA fragments, ligation and transformation of *E. coli* cells were carried out as described [16]. *E. coli* strain W3110lacIQ was used as bacterial host for expression of rPP4 and rPP4-X as described [17]. Nucleotide sequencing was performed according to Maxam & Gilbert [18] or by using the dideoxy chain-terminating method as described by Sanger *et al.* [19].

Fermentation and expression of rPP4 and rPP4-X

For fermentation of rPP4- and rPP4-X-expressing bacterial strains, the following medium was used: 20 g of yeast extract/litre, 138 mM-lactose, 4.7 mM- $\text{NaH}_2\text{PO}_4 \cdot 7\text{H}_2\text{O}$, 48 mM- $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, 13 mM-KCl, 8 mM- $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 6.7 mM-citric acid, 38 mM- $(\text{NH}_4)_2\text{SO}_4$, 0.56 mM- $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, 1.2 μM - $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$, 7.3 μM - ZnCl_2 , 0.04 μM - $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 0.65 μM - $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$, 40 μM - H_3BO_3 , 7.6 μM - $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 3 μM -KI and 5 mg of thiamin/l.

Fermentation of rPP4- and rPP4-X-expressing *E. coli* strains was performed in 1.5 litre B. Braun Biostat M and 10 litre Biostat E fermenters. A single colony of freshly transformed *E. coli* was taken from a Luria broth/agar/Amp plate and inoculated into 100 ml of the fermentation medium supplemented with 50 μg of ampicillin/ml. This preculture was grown in a rotary shaker for 6 h to an A_{650} of 7. The inoculum was 10 ml of preculture/litre of fermentation broth. rPP4 or rPP4-X expression was induced by the addition of IPTG to a final concentration of 1 mM or by the addition of lactose. The pH was adjusted to 7.0 by the addition of NH_4OH (25%). Fermentation employing IPTG induction used a limited glucose concentration in order to avoid catabolite repression. In some experiments 10 ml portions of the fermentation broth were withdrawn from the fermenter at 1 h intervals, bacterial cells were lysed as described [14], cell debris was removed by centrifugation in an SS34 Sorvall rotor for 10 min at 10000 rev./min, and the protein content in the supernatant was analysed by SDS/PAGE on 15% gels. After 6 h of growth and an additional 16–24 h of induction, fermentation was terminated and cells were harvested by centrifugation. The cells were resuspended in the original volume in 0.02 M-Tris/HCl (pH 7.5)/0.01 M-EDTA. The slurry was passed twice through a French press with a pressure difference of 8×10^7 Pa and a flow rate of 40 l/h. The cellular debris was removed by centrifugation. The supernatant was passed through a 0.45 μm filter to remove unlysed bacteria and cell debris.

Purification procedure for rPP4

After addition of Triton X-100 to a final concentration of 1%, the solution (1 litre), which had been purified from cell debris,

Fig. 1. Purification schemes for rPP4 (a) and rPP4-X (b)

was incubated batchwise with DEAE-Sephacrose (300 ml), equilibrated in 0.02 M-Tris/HCl (pH 7.5)/0.01 M-EDTA/0.1% Triton X-100 for 2 h. The resin was washed with 0.02 M-Tris/HCl, pH 7.5 (buffer A), and was packed into a column. Bound proteins were eluted with 0.5 M-NaCl in buffer A, and the ionic strength was lowered by dialysis against buffer A. After addition of CaCl_2 (5 mM) the DEAE-Sephacrose eluate (200 ml) was incubated batchwise with heparin-Sephacrose (150 ml) which had been equilibrated with buffer A containing 5 mM- CaCl_2 for 2 h. Unbound proteins were removed by washing the resin, and adsorbed proteins were eluted with 0.5 M-NaCl in buffer A. EDTA (1 mM) was added to the dialysed eluate (100 ml), which afterwards was pumped through a column of heparin-Sephacrose (100 ml) in buffer A containing 1 mM-EDTA. Unbound protein was collected (120 ml) and was dialysed against buffer A containing 0.15 M-NaCl. All procedures were carried out at 4 °C. The purification schemes for rPP4 and rPP4-X are depicted in Fig. 1.

Purification of r-PP4-X

Lysis of *E. coli* and removal of cell debris was performed as in the PP4 purification technique. In contrast with the isolation procedure of PP4, which was adsorbed on to DEAE-Sephacrose and was collected in the eluate, rPP4-X was found to be present in the DEAE supernatant (1.3 litres) under the conditions used. Except for this difference the following adsorptions for both recombinant proteins were identical.

Purification of PP4 and PP4-X from human placenta was performed according to Bohn *et al.* [6] and Tait *et al.* [12] respectively.

Assay for anticoagulant activity

The anticoagulant activities of PP4, rPP4, PP4-X and rPP4-X were determined using a modified thromboplastin time test: 50 μl of citrated plasma (standard human plasma) was mixed with 150 μl of 0.05 M-Tris/HCl (pH 7.5)/0.15 M-NaCl, 25 μl of buffer or sample and 25 μl of calcium-free thromboplastin. The mixture was incubated for 3 min at 37 °C. Coagulation was initiated by addition of 25 μl of a solution containing 0.02 M- CaCl_2 . The time point of coagulation was determined using a coagulometer according to Schnitzler & Gross [20]. Reference

curves were established by using PP4 and PP4-X which had been isolated from human placenta.

Determination of phospholipase A₂ *in vitro*

The activity of pancreatic phospholipase A₂ *in vitro* was determined by a modification of the procedure described in [21]. Briefly, a volume of 0.4 ml containing 31.25 mM-Tris/HCl, pH 8.0, 12.5 mM-CaCl₂, 900 nM-L-palmitoyl-2-[1-¹⁴C]arachidonoylphosphatidylcholine (54.5 mCi/mmol) and 0.2 units of phospholipase A₂ was incubated with inhibitor at 37 °C for 10 min. The reaction was started by the addition of enzyme and terminated by the addition of 0.1 ml of an aqueous Triton X-100 solution (5%, v/v) containing EDTA (0.2 M) and 25 nmol of [5,6,8,9,11,12,14,15-³H]arachidonic acid (100 Ci/mmol), and subsequently the whole volume was transferred to a solution of 257 mg of ammonium sulphate in 7 ml of cyclohexane. Controls were run without enzyme and/or inhibitor. After thorough mixing and centrifugation in a benchtop centrifuge, 2 ml of the supernatant was used for measuring radioactivity in a scintillation counter. The extraction yield was calculated from the ³H counts. The enzymic activity was determined by measuring ¹⁴C and correcting the values by the extraction yield.

Cellular phospholipase A₂ activity was determined in a human platelet system by measuring the arachidonic acid released as well as the subsequently formed platelet eicosanoids 15-HETE and thromboxane (TX). To enhance the specificity and sensitivity of a determination of these products, the steps of an h.p.l.c. procedure published by Watkins & Peterson [22] were combined with the principles of a fluorimetric assay reported recently [23].

Citrated platelet-rich human plasma [24] containing 70 mM-glucose (2.8 × 10⁵ platelets/μl) was centrifuged in a swinging-bucket rotor at 1000 g for 10 min (4 °C). The pellet was resuspended in PM 16 to a final concentration of approx. 5 × 10⁵ platelets/μl. After addition of CaCl₂ and MgCl₂ (2 mM each), samples of 0.5 ml of the platelet system were incubated with inhibitors (PP4, rPP4, PP4-X or rPP4-X) for 10 min at 37 °C with or without the presence of 100 μM-BW 755c. Platelets were stimulated subsequently with 1 unit of thrombin and the incubation was continued for another 5 min. After termination with 10 μl of HCl (1 M), addition of standard compounds such as oadecatetraenoic acid (ODTA) (2 μg), 15-HETE (1 μg) and PGF_{2α} (1 μg) and extraction with ethyl acetate, the organic phase was dried under a stream of N₂ and assayed as described in the next section.

Separation and derivatization of arachidonic acid

The residues of the dried ethyl acetate extracts were redissolved in n-hexane and passed through pre-equilibrated columns (Supelclean LC-NH₂, 1 ml) washed with n-hexane followed by chloroform/propan-2-ol (2:1, v/v). Arachidonic acid and ODTA were then eluted with diethyl ether containing 2% acetic acid. After removal of the solvent, the residual fatty acids were derivatized with 4-bromo-methyl-7-methoxycoumarin (BMCM) to the corresponding methylmethoxycoumarin (MMC) esters under anaerobic conditions by addition of 5 mg of K₂CO₃ and 50 μl of BMCM solution (1.2 mg/ml of acetonitrile) and subsequent incubation at room temperature for 1 h. Samples were immediately assayed by h.p.l.c. as described below.

Separation and derivatization of eicosanoids

The residues of the ethyl acetate extraction were dissolved in diethyl ether/light petroleum (b.p. 30–60 °C) (1:3, v/v) and filtered on a conditioned LC-Si column. After washing the column with 2 ml of diethyl ether/light petroleum (1:3, v/v), 12-HETE was eluted with 3 ml of diethyl ether/light petroleum (3:1, v/v) and subsequently the prostaglandin fraction (TXB₂)

was eluted with 3 ml of ethyl acetate/methanol (9:1, v/v). After drying under N₂ the fractions were derivatized as described above for arachidonic acid.

H.p.l.c. procedures performed using a Nucleosil C18 column (100 mm × 3 mm). The solvent consisted of 625 ml of acetonitrile and 375 ml of water, and the column was eluted at 0.7 ml/min for the separation of the 12-HETE MMC esters, whereas for the arachidonic acid derivative 800 ml of acetonitrile and 200 ml of water was used, with an elution rate of 1.5 ml/min. The prostaglandin derivatives were eluted using a 100 mm × 4.6 mm Nucleosil C18 column with a solvent system of 450 ml of acetonitrile and 550 ml of water (elution rate 1.5 ml/min).

Physicochemical protein characterization

SDS/PAGE of proteins was performed according to Laemmli [25] and Western blotting analysis according to Towbin *et al.* [26]. Isoelectric points of the purified proteins were determined by chromatofocusing using a Mono P HR 5/20 column as described by the manufacturer. Samples were dialysed against a buffer of 0.025 M-Bistris, pH 7.1, containing 0.025 M-imino-diacetic acid and were pumped on to the column. Elution was performed in Polybuffer 74, pH 4.0, containing 0.025 M- iminodi-acetic acid. Total protein concentration was determined using the BCA protein assay. Gel-permeation chromatography for molecular mass determination of the native proteins was performed on Superose TM 12 HR 10/30 in a buffer of 0.02 M-Tris/HCl (pH 7.5)/0.15 M-NaCl.

Immunological identification and comparison between placental and recombinant proteins was performed by the double-diffusion immunoprecipitation method according to Ouchterlony [27]. Antibodies against each of PP4, rPP4, PP4-X and rPP4-X were raised in rabbits as described by Bohn *et al.* [6] and were purified by affinity chromatography with the appropriate Sepharose-coupled protein. Immunological determination in solution was performed by e.l.i.s.a. according to Engvall & Perlmann [28].

RESULTS

Microbial expression of rPP4 and rPP4-X

The cloning from human placental gene libraries and the characterization of the cDNA for PP4 and PP4-X have been described previously [8,12]. PP4 and PP4-X cDNA was cloned into the expression vector pTrc99A [14], which utilizes the IPTG-inducible *trc* promoter [15] for the high-level expression of cloned genes in *E. coli*. Under fermentation conditions, the expression levels of rPP4 directed by pTrc99A-PP4 and of rPP4-X directed by pTrc99A-PP4-X were identical. In general, 2–3 g of the recombinant fully soluble protein was present in a 1 litre fermenter at the time of termination of the fermentation. Fig. 2 shows a typical course of a rPP4/rPP4-X fermentation run. In this example, *E. coli* W31101acQL8 strain (pTrc99A-PP4) was fermented and the *trc* promoter was induced by the addition of IPTG at an absorbance corresponding to approx. 15 g dry weight/litre (at time point zero). During the following 24 h of fermentation the steady-state yield of PP4 increased constantly, and it constituted the most prominent protein band at later times. At this point, 24 h after induction, the fermenter contained a biomass of approx. 60 g dry weight and the fermentation run was terminated. The cells were collected and lysed as described in the Materials and methods section. After removal by centrifugation of residual unlysed cells and cellular debris, rPP4 and rPP4-X were found almost exclusively in the supernatants. Only very small amounts of these proteins were present in the pellet fraction, as determined by SDS/PAGE and Western-blot

Fig. 2. Product formation of rPP4 during *E. coli* fermentation

A 1 litre *E. coli* W3110lacIQL8 (pTrc99A-PP4) culture was fermented as described in the Materials and methods section. At an absorbance corresponding to 15 g dry weight/l (time 0 in the Figure), the *trc* promoter was induced by the addition of IPTG (1 mM final concentration). At time points 0, 1, 2, 3, 4, 5, 6, 9, 12 and 24 h after induction, 10 ml portions were withdrawn from the fermenter and frozen at -80°C . Bacteria were lysed as described [14], cell debris was removed by centrifugation and the proteins present in the supernatants were analysed on SDS/PAGE (15% gels). The gel was stained with Coomassie Blue. For comparison a purified PP4 sample is shown (lane S). Molecular masses of the standards are shown (lane M).

analyses (results not shown). From these results it is obvious that rPP4 and rPP4-X are fully soluble and that even after very high expression these proteins do not form insoluble inclusion bodies.

In order to investigate the stability of rPP4 and rPP4-X in the *E. coli* lysates, such lysates were prepared and stored at 4°C for up to 3 weeks. Losses of the recombinant proteins only amounted to 5–10%, as determined by e.l.i.s.a. and Western-blot analyses.

From these results it is evident that both rPP4 and rPP4-X can be expressed at very high levels in a soluble form in *E. coli*, and that these proteins are also very stable in *E. coli* lysates for long time periods.

Expression of a PP4 mutant protein

PP4 already has been expressed and characterized by other investigators [8–10]; these reports, however, did not give details on protein stability. For further investigation of protein stability, we expressed in *E. coli* a shortened form of rPP4, named rPP4-delta, in which 13 C-terminal amino acids of rPP4 were deleted. Only 5% of this truncated protein could be detected in *E. coli* lysates compared with the expression rate of rPP4 and, moreover, PP4-delta was only partly solubilized by detergent (results not shown). Therefore we assume that the C-terminus of PP4 is important for the solubility and/or the stability of this protein.

Purification of rPP4

E. coli lysates were treated with EDTA and Triton X-100 to solubilize all of the recombinant proteins. The presence of detergent turned out to be essential for binding of rPP4 to DEAE-Sepharose. In the absence of Triton, only 20% of PP4 was adsorbed. Although this adsorption step did not achieve a good enrichment (Fig. 3a), total protein was concentrated, and Triton and EDTA could be simply removed from the proteins by washing the resin. Bound proteins were eluted by raising the ionic strength. After addition of CaCl_2 the diffusate was incubated with heparin-Sepharose. No residual rPP4 could be detected in the supernatant, and adsorbed proteins were eluted with 0.5 M-NaCl. The main bacterial impurities were removed by this chromatography. The dialysed eluate was pumped through a column of heparin-Sepharose in the presence of EDTA. Under these conditions more than 95% of rPP4 passed through the resin unbound. The rest of the bacterial proteins were adsorbed. EDTA was removed from the > 95% pure rPP4 by dialysis.

Purification of rPP4-X

Addition of Triton and EDTA also turned out to be important

Fig. 3. SDS/PAGE of the recombinant proteins after various purification steps

(a) Isolation of rPP4: lanes 1 and 7, BRL molecular mass markers (values in kDa); 2, crude *E. coli* extract; 3, DEAE-Sepharose eluate; 4, pool after heparin-Sepharose (CaCl_2); 5, pool after heparin-Sepharose (EDTA); 6, PP4 purified from human placenta. (b) Isolation of rPP4-X: lanes 1 and 7, BRL molecular mass markers (values in kDa); lane 2, crude *E. coli* extract; 3, supernatant after incubation with DEAE-Sepharose; 4, heparin-Sepharose (CaCl_2) eluate; 5, pool after heparin-Sepharose (EDTA); 6, PP4-X purified from human placenta. Reducing agent was added to the sample buffer.

for solubilizing rPP4-X. Under the chosen conditions rPP4-X passed through the DEAE-Sephacrose unbound. Since a large number of bacterial proteins were bound to the resin, this process resulted in enrichment of rPP4-X (Fig. 3b). After dialysis of the supernatant, rPP4-X was adsorbed on to heparin-Sephacrose in the presence of CaCl_2 . Triton was removed by washing the resin. The final purification steps were identical with those in the rPP4 isolation procedure and rPP4-X of greater than 95 % purity was obtained.

Compared with the starting material in the crude *E. coli* lysates, the overall yields of 95 % pure proteins were 30 % for rPP4 and 15 % for rPP4-X.

Characterization of the recombinant proteins

In order to compare the physicochemical properties of the recombinant proteins with their placental counterparts, molecular masses, isoelectric points and immunological identity were investigated. Molecular masses of the native proteins were determined by gel-permeation chromatography. Each protein was eluted as a monomer in a single sharp peak, revealing molecular masses of 33–36 kDa for PP4, rPP4, PP4-X and rPP4-X (results not shown). Identical apparent sizes were also observed as Coomassie Blue-stained bands of the purified proteins from different sources in SDS/PAGE (Fig. 3). Comparison of the isoelectric points (pI) showed that rPP4 (pI 4.9 ± 0.1) was slightly different from the placental protein (pI 4.8 ± 0.1). The same was observed for rPP4-X (pI 6.1 ± 0.1) compared with PP4-X (pI 6.0 ± 0.1).

N-Terminal sequence determination of rPP4 revealed the absence of the N-terminal methionine (results not shown). Immunological identity of the recombinant proteins with their placental counterparts could be demonstrated by immunoprecipitation employing the Ouchterlony immune diffusion test (Fig. 4). Antibodies raised against PP4 or rPP4 did not cross-react with PP4-X or rPP4-X and vice versa. Purified PP4 (PP4-X) or rPP4 (rPP4-X) ($10 \mu\text{g}$ of each) was added to *E. coli* control lysates (1 ml) lacking the recombinant proteins. Both the placental as well as the recombinant proteins were recognized quantitatively by the antibodies to the full extent, as determined by e.l.i.s.a. Again, no cross-reactions between PP4 (rPP4) and PP4-X (rPP4-X) were observed (results not shown).

Biological activities of the recombinant proteins compared with the placental proteins were investigated by their ability to inhibit both the clotting activity of human plasma and the release of arachidonic acid by phospholipase A_2 .

Anticoagulant properties

Using standard human plasma, increasing amounts of PP4, rPP4, PP4-X or rPP4-X were added and the time point of

Fig. 4. Immunoprecipitation of PP4 and PP4-X

Immunoprecipitation of PP4 (cavities 1 and 6), rPP4 (2 and 4) and rPP4-X (5) is shown with antibodies raised against PP4 (7) and PP4-X (8).

Fig. 5. Concentration-dependent inhibition of clotting by placental and recombinant proteins

Inhibition was determined by using a modified thromboplastin time test as described in the Materials and methods section. Symbols: *, PP4; Δ , rPP4; $\circ$, PP4-X; $\square$, rPP4-X.

Fig. 6. Inhibition of phospholipase A_2 -catalysed release of arachidonic acid *in vitro* by increasing amounts of added placental or recombinant proteins

*, PP4; Δ , rPP4; $\circ$, PP4-X; $\square$, rPP4-X.

clotting was determined by the modified thromboplastin time test, as described in the Materials and methods section. As shown in Fig. 5, both purified recombinant proteins rPP4 and rPP4-X showed about 90–95 % of the anti-clotting activity of their placental counterparts. This experiment also showed that PP4/rPP4 is a more effective coagulation inhibitor than PP4-X/rPP4-X. As expected, neither PP4/rPP4 nor PP4-X/rPP4-X influenced the 'thrombin time' (results not shown).

Inhibition of arachidonic acid release by phospholipase A_2

The investigated proteins markedly inhibited the release of arachidonic acid by pancreatic phospholipase A_2 *in vitro* in a concentration-dependent way, as demonstrated in Fig. 6. These experiments showed that in this test system, too, PP4/rPP4 is the more potent inhibitor. The inhibitory effects on thrombin-stimulated human platelets are summarized in Tables 1 and 2. Thrombin stimulated the release of arachidonic acid, which in platelets is the precursor for 12-HETE and TXA_2 . Under normal conditions the released arachidonic acid is immediately metabolized to these eicosanoids, and no free arachidonic acid

Table 1. Inhibitory effects of PP4 and rPP4 on thrombin-induced 12-HETE and TXB₂ release in human platelets *in vitro*

Values are shown as percentages of control values (thrombin alone) and are means $\pm$ S.E.M. ($n = 6$) for 12-HETE and means for TXB₂. n.d., not determined.

Addition	Release (% of control)	
	12-HETE	TXB ₂
Thrombin (1 unit) (control)	100 $\pm$ 3	100
PP4 (2 mg/ml) + thrombin	56 $\pm$ 15	n.d.
rPP4 (2 mg/ml) + thrombin	59 $\pm$ 10	56
None (without thrombin)	1 $\pm$ 0	n.d.

Table 2. Inhibitory effects of PP4, PP4-X and rPP4 on thrombin/BW 755c-induced accumulation of arachidonic acid in human platelets *in vitro*

Values are shown as percentages of control values (addition of thrombin alone), and are means $\pm$ S.E.M., with numbers of experiments in parentheses. For details see the Materials and methods section.

Addition	Arachidonic acid accumulation (% of control)
Thrombin (1 unit) (control)	100 $\pm$ 1 (17)
PP4 (2 mg/ml) + thrombin	68 $\pm$ 5 (6)
rPP4 (2 mg/ml) + thrombin	66 $\pm$ 2 (6)
PP4-X (2 mg/ml) + thrombin	93 $\pm$ 4 (6)
None (without thrombin)	2 $\pm$ 0.1 (18)

can be detected. However, blocking the lipoxygenase enzyme catalysing 12-HETE formation, as well as cyclo-oxygenase, which catalyses TXA₂ formation, by the addition of the dual inhibitor BW 755c led to an accumulation of arachidonic acid which was quantified by the applied fluorescence method. As shown in Tables 1 and 2, the investigated proteins are able to suppress the formation of 12-HETE and of TXA₂, as well as causing the accumulation of arachidonic acid.

DISCUSSION

In the present study we have expressed the human placenta proteins PP4 and PP4-X in *E. coli*. Subsequently, both recombinant proteins were purified to homogeneity, physico-chemically characterized and compared with their placental counterparts.

Cultivation of the PP4- and PP4-X-expressing bacterial strains and IPTG induction under optimized fermentation conditions resulted in the expression of very high levels of both proteins: approx. 2–3 g of biologically active rPP4 or rPP4-X was present in a 1 litre fermenter. Both proteins were found to be expressed in a soluble form. Their presence is non-toxic for the bacterial host, since the rPP4/rPP4-X-expressing bacterial cultures grew with normal kinetics. Microscopic inspection of induced individual bacterial cells did not reveal any morphological differences compared with non-expressing control cells. Moreover, rPP4 and rPP4-X were found to be very stable in bacterial lysates.

On purification starting from *E. coli* lysates, > 95% pure rPP4 and rPP4-X were obtained, with yields of 30% and 15% respectively. All purification steps can be performed in batches;

ion-exchange- or affinity-resin-adsorbed proteins were eluted by high ionic strength solutions without a salt gradient. Therefore these processes seem to be suitable for large-scale purification of these recombinant proteins. The most effective enrichment of rPP4, and to a lesser extent of rPP4-X, was obtained by adsorption to heparin-Sepharose. We used the property of these proteins of binding to this affinity resin in the presence of calcium; only a small fraction of total bacterial protein was adsorbed under these conditions. For final purification this chromatography was repeated in the presence of EDTA, and again only bacterial proteins were bound to the resin. The other very effective purification step for rPP4-X was DEAE-Sepharose adsorption, since this step removed many contaminating bacterial proteins. Despite the presence of detergent and chelating agent, rPP4-X was not bound to the immobilized DEAE, although the placental protein purified by the method of Tait *et al.* [12] did bind. A possible explanation is that rPP4-X is associated with molecules of bacterial origin.

Comparison of the placental proteins PP4 and PP4-X with their recombinant counterparts showed physicochemical identity between them. Only the isoelectric points of the recombinant proteins showed a slight shift, as described previously for rPP4 [11]. Maurer-Fogy *et al.* [9] reported that this difference was due to the unblocked N-terminal alanine residue of the recombinant VAC which also holds true for rPP4 and rPP4-X. The recombinant and placental proteins also had immunological identity. Despite the great sequence similarity of PP4 and PP4-X, no cross-reactivity was found, as has already been described for the placental proteins [13]. This result shows that immunodominant regions are most probably located outside the conserved protein domains.

The placental and recombinant proteins also showed identical anticoagulant activities. Determination of anti-clotting activities showed that at least 90–95% of purified rPP4 and rPP4-X was active in these tests. Modified thromboplastin time, plasma recalcification time (results not shown) and the phospholipase A₂ reaction were inhibited by these proteins in a concentration-dependent manner, due to their ability to bind to phospholipid vesicles or membranes in a calcium-dependent manner [5,29]. Release of arachidonic acid by phospholipase A₂, however, was significantly less inhibited (in the lower concentration range) by rPP4-X than by the placental protein, a result which we cannot explain at the present time. Comparison of the inhibitory potency of the annexins in a cell-free system *in vitro* or in a platelet test system clearly shows that arachidonic acid is released to a much higher extent in the platelet system. This might be due to the fact that extracellular PP4 or PP4-X is able to influence the fatty acid release reaction only to a much lower extent than from inside the cell. Nevertheless, a significant decrease in arachidonic acid release can be achieved by extracellular application of annexins. This has also been reported by other investigators [30–32]. This effect may be caused by signal transduction, occurring after annexin binding to the extracellular side of the plasma membrane or from the penetration or internalization of the annexins. A satisfactory explanation, however, remains to be found.

In agreement with the results of Tait *et al.* [12], who described PP4 and PP4-X as PAP I and PAP II respectively, we also found that PP4 (rPP4) is the more potent inhibitor in both functional tests.

Investigations on other functional properties of the annexins, such as binding to elements of the cytoskeleton [33] or participation in exocytosis [34] will hopefully provide a more detailed insight into the physiological role of this interesting protein family.

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