Human Placental Anticoagulant Protein: Isolation and Characterization†

Takayuki Funakoshi,‡ Ronald L. Heimark,§ Lee E. Hendrickson,‡ Brad A. McMullen,‡ and Kazuo Fujikawa*,‡
Departments of Biochemistry and Pathology, University of Washington, Seattle, Washington 98195
Received March 25, 1987; Revised Manuscript Received April 16, 1987

ABSTRACT: An anticoagulant protein was purified from the soluble fraction of human placenta by ammonium sulfate precipitation and column chromatography on DEAE-Sepharose, Sephadex G-75, and Mono S (Pharmacia). The yield of the purified protein was approximately 20 mg from one placenta. The purified protein gave a single band by sodium dodecyl sulfate–polyacrylamide gel electrophoresis with a moelcular weight of 36 500. This protein prolonged the clotting time of normal plasma when clotting was induced either by brain thromboplastin or by kaolin in the presence of cephalin and Ca^{2+}. It also prolonged the factor Xa induced clotting time of platelet-rich plasma but did not affect thrombin-induced conversion of fibrinogen to fibrin. The purified placental protein completely inhibited the prothrombin activation by reconstituted prothrombinase, a complex of factor Xa–factor Va–phospholipid–Ca^{2+}. The placenta inhibitor had no effect on prothrombin activation when phospholipid was omitted from the above reaction. Also, it neither inhibited the amidolytic activity of factor Xa, nor did it bind to factor Xa. The placenta inhibitor, however, did bind specifically to phospholipid vesicles (20% phosphatidylserine and 80% phosphatidylcholine) in the presence of calcium ions. These results indicate that the placental anticoagulant protein (PAP) inhibits coagulation by binding to phospholipid vesicles. The amino acid sequences of three cyanogen bromide fragments of PAP aligned with those of two distinct regions of lipocortin I and II with a high degree of homology, showing that PAP is a member of the lipocortin family.

The endothelium is important in maintaining blood fluidity by preventing blood coagulation (Florey, 1966; Mason et al., 1977). Glycosaminoglycan in the endothelial cell plasma membrane has been thought to prevent activation of blood coagulation (Mason et al., 1977). A heparin-like compound, which is a potent anticoagulant, has been localized on the surface of cultured endothelial cells (Marcum et al., 1984). Anticoagulant activity associated with thrombomodulin is another antithrombogenic mechanism provided by endothelial cells (Owen & Esmon, 1981). Thrombomodulin forms a complex with thrombin, and this complex activates protein C (Esmon et al., 1985), which terminates coagulation by inactivating factor VIIIa (Vehar & Davie, 1980) and factor Va molecules (Kisiel et al., 1977).

Recently, injured or activated endothelial cells have been shown to initiate coagulation (Stern et al., 1985). Tissue factor is also known to be present in endothelial cells (Maynard et al., 1977), and its synthesis and expression on the cell surface are stimulated by the perturbation of the cells (Nawroth et al., 1986; Bevilacoqua et al., 1986).

The results indicate that cellular components participate in both thrombostatic and thrombotic events under physiological conditions. We wondered whether any other cellular protein(s) was (were) involved in regulating the coagulation system. We looked for such a protein(s) in cultured bovine aortic endothelial cells and found an anticoagulant activity in the soluble fraction of the cell homogenate. This activity was adsorbed to DEAE-Sepharose and eluted from a gel filtration column in a molecular weight range of 30 000–40 000. However, limited amounts of the cells made it difficult to purify this activity. We then chose human placenta as a substitute source of this protein and have continued the purification of a protein responsible for this anticoagulant activity. Previously, human placenta had been shown to be a useful source in purification of thrombomodulin (Salem et al., 1984). In this paper, we describe the isolation and characterization of the placental anticoagulant protein, tentatively called "PAP",[1] which is similar or possibly the same as the endothelial anticoagulant protein. The ongoing sequence analysis of PAP revealed that this protein was a new member of the "lipocortin" or "calpactin" family.

Experimental Procedures

Assay of Clotting Activity. Reagents: The complete contents of one vial of rabbit brain cephalin (Sigma) was uniformly suspended in 100 mL of saline and stored in 1-mL aliquots at −20 °C. Equal volumes of the cephalin suspension and 33 mM $CaCl_2$ were mixed before each assay. Acid-washed kaolin (Fischer) was suspended in saline at 50 mg/mL. Assay procedure: The anticoagulant activity of PAP was determined in three different systems using normal human plasma. (1) Inhibition of the intrinsic coagulation pathway: 20 μL of plasma, 20 μL of kaolin, and 10 μL of a test sample in buffer A[2] were incubated for 10 min at 37 °C. Then, 40 μL of $CaCl_2$–cephalin mixture were added, and the clotting time was determined. (2) Inhibition of the extrinsic coagulation pathway: the clotting time was determined by the same method as described above except that kaolin suspension was replaced by thromboplastin. Human brain thromboplastin was diluted with 50 mM Tris-HCl, pH 7.4, containing 0.15 M NaCl to obtain a control clotting time of approximately 60

† This work was supported by Research Grant HL 16919 from the National Institutes of Health.
* Address correspondence to this author.
‡ Department of Biochemistry.
§ Department of Pathology.

[1] Abbreviations: AMC, 7-amino-4-methylcoumarin; BSA, bovine serum albumin; CM, *S*-(carboxymethyl); EDTA, ethylenediaminetetraacetic acid; MCA, 4-methylcoumarinamide; PAP, placental anticoagulant protein; PC, phosphatidylcholine; PS, phosphatidylserine; SDS, sodium dodecyl sulfate; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

[2] The components of the buffers are as follows: buffer A, 50 mM Tris-HCl, pH 7.9, containing 50 mM NaCl; buffer B, 50 mM Tris-HCl, pH 7.9, containing 0.2 M NaCl; buffer C, 25 mM sodium acetate, pH 5.2; buffer D, 0.5 M NaCl in buffer C.

0006-2960/87/0426-5572$01.50/0

s. (3) Inhibition of clotting activity of platelet-rich plasma induced by factor Xa: 5 ng of factor Xa in 5 μL of buffer A was mixed with 10 μL of test sample in buffer A for 3 min at room temperature; 20 μL of platelet-rich plasma and 20 μL of 33 mM $CaCl_2$ were then added, and the clotting time was determined. The effect of PAP on the clotting activity of thrombin was tested with the thrombin assay method of Seegers (1964). Thrombin (10 μL), of which the concentration was adjusted to obtain a control clotting time of 55 s, was incubated for 3 min at 37 °C with 10 μL of various concentrations of PAP. The sample was then added to a mixture of 20 μL of 1% fibrinogen and 60 μL of gum arabic solution, and the clotting time was recorded.

The effect of PAP on prothrombinase activity was studied as follows: 1.1 nM factor Xa (5 ng), 9.5 nM factor Va (0.25 μg), 8.9 μM prothrombin (50 μg), and 0.1 μg of phospholipid (PS/PC, 20/80) were incubated in 80 μL of buffer A containing BSA (0.1 mg/mL) for 3 min at room temperature in the presence or absence of PAP. The activation of prothrombin was initiated by the addition of 4 μL of 0.1 M $CaCl_2$, and the reaction was terminated after 2 min by the addition of 5 μL of 0.5 M EDTA. The amounts of thrombin generated were assayed by the amidolytic activity with Boc-Val-Pro-Arg-MCA (Morita et al., 1977). Ten microliters of the above reaction mixture was diluted with 1 mL of buffer A containing BSA (0.1 mg/mL), and 2 μL of the diluted sample was assayed for the thrombin activity. From the reactions conducted without phospholipid or factor Va, 2 μL of the reaction mixture was assayed directly for the thrombin activity. These samples were then incubated for 5 min at 37 °C with 0.1 mL of 0.5 mM substrate. The reactions were stopped by the addition of 2 mL of 10% acetic acid. Intensity of fluorescence was determined by a Perkin-Elmer LS-5 fluorescence spectrometer with excitation at 380 nm and emission at 460 nm. Thrombin activity was expressed as the amounts of AMC formed per minute in the prothrombin activation mixture.

Amidolytic activity of factor Xa was determined as described above. Five nanograms of factor Xa, 20 μg of BSA, and a test sample were incubated for 3 min at 37 °C in 35 μL of buffer A, and 0.1 mL of 0.5 mM Boc-Ile-Glu-Gly-Arg-MCA was then added. After incubation for 60 min, intensity of fluorescence was determined as above.

Western blot analysis was performed according to Towbin et al. (1979). Proteins were electrophoresed on a 7.5% SDS gel (Laemmli, 1970) and then transferred electrophoretically to nitrocellulose, and bound antigens were detected by ^{125}I-labeled affinity purified anti-PAP.

Binding of PAP to phospholipid vesicles were measured by gel filtration. Phospholipid vesicles were prepared as follows: 0.4 mg of egg yolk PC and 0.1 mg of bovine brain PS in chloroform were mixed in a test tube, and the organic solvent was evaporated under N_2 gas. The dried lipid was suspended in 0.5 mL of 50 mM Tris, pH 7.4, containing 0.15 M NaCl, and the suspension was sonicated twice for 15 s. A complete binding mixture contained in 220 μL of buffer A 0.1 mg of phospholipid vesicles, 5 μg of ^{125}I-PAP (total 2.5×10^5 cpm), BSA (1 mg/mL), and 5 mM $CaCl_2$. This mixture was applied to a column (0.7×14 cm) of Sepharose 4B equilibrated with buffer A. Fractions of 0.5 mL were collected at a flow rate of 5 mL/h. The concentrations of phospholipid vesicles, PAP, and BSA in the fractions were determined by 90° light scattering (320 nm), radioactivity, and Bradford protein analysis, respectively. Binding of PAP to factor Xa, factor Va, or prothrombin was tested as follows: 5 μg of ^{125}I-PAP (8000 cpm) was mixed with either factor Xa (5 μg), factor Va (2.5 μg), or prothrombin (10 μg) in 0.1 mL of buffer A containing 5 mM $CaCl_2$. The mixture was applied to a gel filtration column (7.5×600 cm) of UltroPac TSK-G 3000 SW connected to a Waters HPLC system. Gel filtration was performed at a flow rate of 1 mL/min with 5 mM Tris-HCl, pH 7.2, containing 50 mM NaCl and 1 mM $CaCl_2$. The protein concentration was monitored by absorbance at 214 nm, and PAP was detected by radioactivity.

SDS–polyacrylamide gel electrophoresis was performed according to Weber and Osborn (1969). Detection of carbohydrate in samples was carried out on an SDS gel with Schiff base staining (Glossmann & Neville, 1971). Concentration of free sulfhydryl groups was quantitated by the Ellman reagent (Ellman, 1959). Protein concentration of PAP was determined by the method of Bradford (1976) with BSA as a standard. A 1% PAP solution determined by this method had a $E_{280}^{1\%}$ value of 6.0. PAP was labeled by reductive methylation (Means & Feeney, 1968) with NaB^3H_4. The labeled protein contained 1.6 mol of ^{3}H/mol with a specific activity of 3×10^7 cpm/mg.

PAP (2 mg) was carboxylmethylated (Crestfield et al., 1963) and digested with cyanogen bromide. The resulting peptides were fractionated on a G-50 superfine column (1.5×28 cm) equilibrated with 4 M guanidine hydrochloride. The pooled fractions were further purified on an Altex Ultrapore RPSC column (4.6×75 mm) connected to a Waters HPLC system. A linear gradient was composed of 0.1% trifluoroacetic acid as the mobile phase and 0.08% trifluoroacetic acid in 80% acetonitrile as the mobile phase modifier. Sequence analysis was performed on a Beckman 890C sequencer, and the phenylthiohydantoin amino acids were identified by two complementary HPLC systems as described previously (McMullen & Fujikawa, 1985).

Antibody to PAP was raised in rabbits by three injections of 0.5 mg of protein over a period of 5 weeks. The immunoglobulin G (IgG) fraction was prepared by the method of Harboe and Ingild (1973) and further purified by an affinity column of PAP immobilized to Affi-Gel 15. The purified antibody was then radiolabeled with $Na^{125}I$ by use of Iodogen (Fraker & Speck, 1975). Human factor X and prothrombin were purified according to Miletich et al. (1981). Factor X was activated by a Russell's viper venom enzyme as described previously (Fujikawa et al., 1972). Platelet-rich plasma was prepared from freshly drawn human blood by centrifugation at 160*g* for 5 min. Purified human factor V was kindly supplied by Dr. W. H. Kane of our laboratory, and it was activated by thrombin. Human thromboplastin was generously given by Dr. W. Kisiel, University of New Mexico. Bovine thrombin was purified from commercial topical thrombin (Parke-Davis) according to Lundblad et al. (1976). Human fibrinogen (76% clottable) was obtained from Calbiochem.

Egg yolk PC (type V-E), bovine brain PS, rabbit brain cephalin, gum arabic, and Ellman reagent were the products of Sigma. NaB^3H_4 (319 mCi/mol) was purchased from New England Nuclear. $Na^{125}I$ (13.6 mCi/μg of iodine) was obtained from Amersham. Iodogen was purchased from Pierce. Affi-Gel 15 was the product of Bio-Rad. Boc-Val-Pro-Arg-MCA and Boc-Ile-Glu-Gly-Arg-MCA were obtained from Peptide Institute Inc., Osaka, Japan.

Results

Purification of PAP. A fresh human placenta was obtained from a local hospital, and the umbilical cord and amnionic membrane were removed. The placenta was then cut into small pieces by a meat chopper and washed in 2 L of cold buffer A to remove the blood. The placenta was then trans-

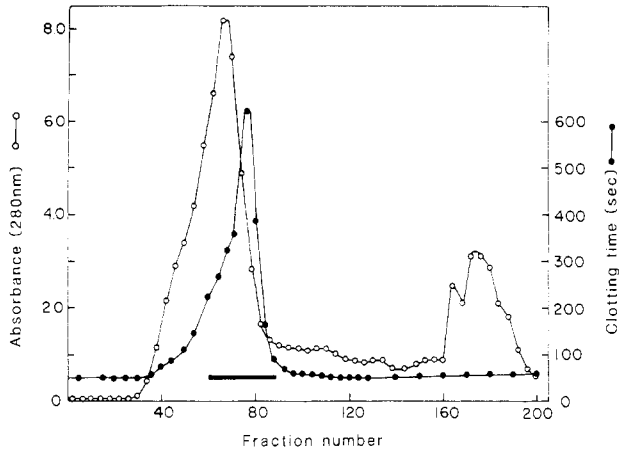

FIGURE 1: Separation of PAP by DEAE-Sepharose column chromatography. The ammonium sulfate fraction was adsorbed to the DEAE column, and proteins were eluted as described under Experimental Procedures. Fractions of 10 mL were collected at a flow rate of approximately 100 mL/h. Anticoagulant activities were determined by inhibition of the intrinsic clotting activity. The active fractions that are shown by a bar were pooled and processed for the next step. Absorption at 280 nm (○); anticoagulant activity (●).

ferred to a Waring blender and homogenized for 1 min with 1 L of cold buffer A containing 5 mM EDTA and 5 mM benzamidine. The homogenate was filtered through a sheet of gauze. Tissue remaining on the gauze was put back into the blender, and homogenization was repeated with 1 L of the same buffer. The homogenate was then filtered, and the two filtrates were combined.

Solid ammonium sulfate was added to the combined filtrates to 40% saturation. Precipitates were removed by centrifugation. Ammonium sulfate was further added to the supernatant to 80% saturation, and the precipitate was collected by centrifugation. The precipitate was then dissolved in 400 mL of buffer A and dialyzed overnight against 12 L of the same buffer with two changes.

The dialyzed sample was transferred to a 4-L plastic beaker and stirred for 2 h with 350 mL of DEAE-Sepharose CL6B gel that had been equilibrated with buffer A. After the gel settled, the supernatant was decanted, the gel was then poured into a plastic column (4.5 × 30 cm), and the column was washed with 2 L of buffer A. Adsorbed proteins were then eluted with a linear gradient formed by 1 L of buffer A and 1 L of buffer A supplemented with 0.45 M NaCl. Fractions were assayed for inhibition of intrinsic coagulant activity as described under Experimental Procedures, and the active fractions that were eluted at the tailing edge of the first protein peak were pooled (Figure 1).

Ammonium sulfate was added to the pooled fraction to 80% saturation, and the precipitate was collected by centrifugation. The precipitate was then dissolved in 50 mL of buffer B and dialyzed for 2 h against 2 L of buffer B. The dialyzed sample was then applied to a column (5.5 × 100 cm) of Sephadex G-75 equilibrated with buffer B. Gel filtration was performed with buffer B at a flow rate of approximately 100 mL/h. An anticoagulant activity appeared after the elution of the major protein peak (Figure 2). The active fractions were pooled and dialyzed against 2 L of buffer C with two changes of buffer.

The dialyzed sample was divided into five aliquots, and each was applied to a Mono S column connected to an FPLC system (Pharmacia). After the column was washed with buffer C, adsorbed proteins were eluted with a linear gradient composed of buffer C and buffer D. Elution was performed by a flow rate of 1.0 mL/min with a 0.67% increment of buffer D per

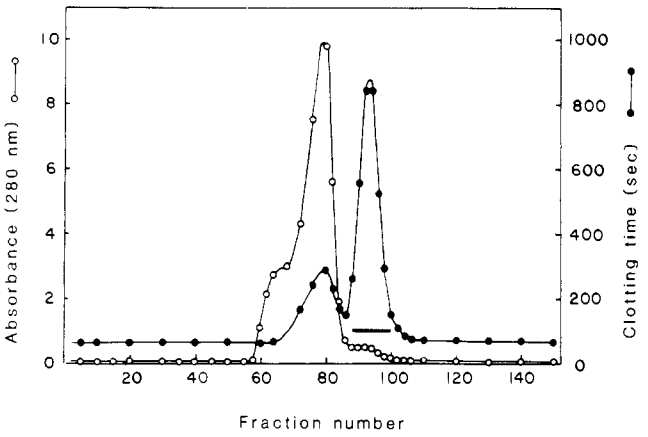

FIGURE 2: Gel filtration of PAP on Sephadex G-75 column. The pooled DEAE-Sepharose fraction was applied to the G-75 column and eluted as described under Experimental Procedures. Fractions of 10 mL were collected. The active fractions that are indicated by a bar were pooled for the next step of purification. Absorption at 280 nm (○); anticoagulant activity (●).

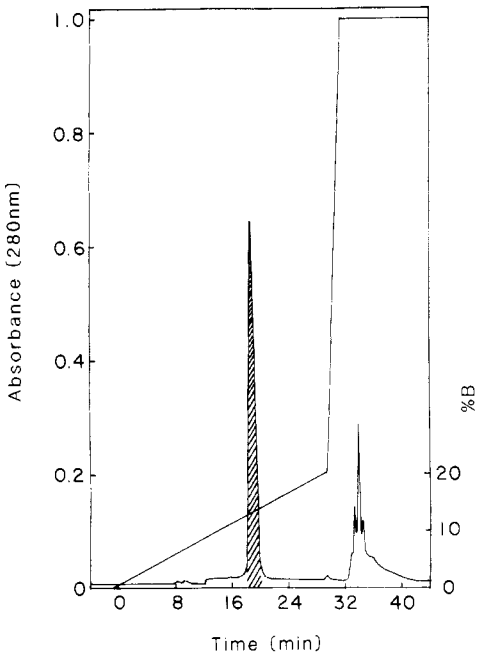

FIGURE 3: Mono S column chromatography of PAP. The sample from the G-75 column was applied to a Mono S column and eluted as described under Experimental Procedures. The fractions in the shaded region contained homogeneous protein and were pooled.

minute. A major protein peak that eluted at approximately 12% of buffer D was collected (Figure 3). This fraction contained a homogeneous anticoagulant protein. Approximately 20 mg of the purified protein was obtained from one placenta by this procedure.

A minor anticoagulant activity, which corresponded to approximately 10% of the major activity, was eluted in the earlier fractions (tubes 68–82) from the gel filtration column (Figure 2). It was possible that this activity was due to the presence of an aggregated or bound form of PAP. Western blot analysis using ^{125}I-labeled antibody against PAP did not detect the antigen in these fractions, indicating the presence of another anticoagulant protein in the DEAE-adsorbed fraction. We have also detected significant amounts of an anticoagulant activity in the nonadsorbed fraction from the DEAE-Sephadex column. These activities are possibly due to lipocortins that have been isolated from human placenta (Huang et al., 1986) (see Discussion).

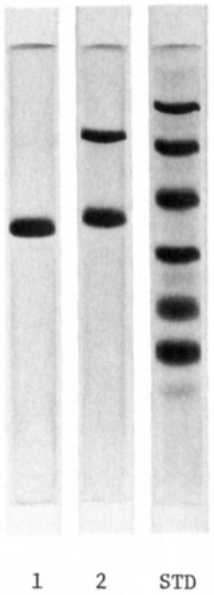

FIGURE 4: SDS–polyacrylamide gel electrophoresis pattern of PAP. Electrophoresis was carried out in 7.5% polyacrylamide gels at 8 mA/tube for 3 h according to Weber and Osborn (1969). Gel 1, 10 μg of reduced PAP; gel 2, 10 μg of nonreduced PAP; gel 3, molecular weight standards, from top, phosphorylase *b* (94 000), BSA (68 000), ovalbumin (43 000), carbonic anhydrase (30 000), soybean trypsin inhibitor (20 000), α-lactalbumin (14 400).

Criteria of Purity and Molecular Weight of PAP. The purified protein gave a single band of a molecular weight of 36 500 on SDS–polyacrylamide gel under reduced condition (Figure 4, gel 1). The homogeneity of the protein was also demonstrated by disc gel electrophoresis. Two bands, which corresponded to molecular weights of 69 000 and 35 000, were observed on SDS gels with nonreduced sample (gel 2). Doubling in the molecular weight observed in the larger band indicated that the larger species was formed by the dimerization of the molecule. The dimer was probably formed by oxidation of a cysteinyl residue(s) present in the protein, because the dimer was not observed in the reduced sample.

Chemical Characterization of PAP. The above experiments suggested the presence of a cysteinyl residue(s) in PAP. Detection of free sulfhydryl groups in the protein was performed with Ellman's reagent in the presence and absence of 6 M guanidine hydrochloride; 0.42 mol/mol of free sulfhydryl group was detected in the native protein, and 0.83 mol was found in the denatured protein. Amino acid analysis of the CM-PAP gave 1.2 mol of CM-cysteine/mol of protein. These results indicate the presence of 1 mol of cysteinyl residue/mol of protein, which is partially buried in the molecule. Exposure of PAP to denaturing agents such as SDS probably made this cysteinyl residue susceptible to oxidation and led to the dimer formation that was observed on the SDS gel. The dimer was not detected in the purified PAP preparation, when the protein was stored for several weeks in buffer A at −20 °C. This result indicates that formation of the dimer is substantially slower or zero under nondenaturing condition.

PAP is probably not a glycoprotein, because staining of an SDS gel sample of PAP with a periodate–Schiff reagent failed to detect carbohydrate.

Amino acid sequence analysis of the intact CM-PAP resulted in no apparent sequence, suggesting that the NH_2 terminus was blocked. Homogeneous peptides isolated from the cyanogen bromide digest were subjected to Edman degradation. The sequences of three of these peptides were found to align with two distinct regions of lipocortins I and II (Figure 5). PAP has 54% identity with lipocortin II and 45% with lipocortin I in these regions. These results clearly show that PAP is a member of the lipocortin family.

Anticoagulant Activities of PAP. Effects of PAP on both intrinsic and extrinsic pathways were examined. Both kaolin- and thromboplastin-induced clotting times were proportionally prolonged by the addition of increasing amounts of PAP to the clotting assay mixtures (Figure 6). The addition of 0.5 μg (at a final concentration of 0.15 μM) of PAP prolonged the kaolin-induced clotting time from 75 to 295 s. Similarly, thromboplastin-induced clotting time was prolonged from 43 to 210 s. PAP also inhibited factor Xa induced clotting time of platelet-rich plasma. A 47-s control clotting time increased to 220 s by the addition of 0.5 μg of PAP. This prolongation corresponded to a greater than 98% inhibition of factor Xa, when it was calculated from a standard calibration curve constructed by serial dilution of factor Xa. PAP, however, did not inhibit the amidolytic activity of factor Xa.

In addition, PAP did not affect the ability of thrombin to convert fibrinogen to fibrin. In these experiments, a control clotting time of 55 s did not change significantly by the addition of up to 3 μg of PAP. The amidolytic activity of thrombin was also not inhibited by PAP.

On the basis of the above results, PAP appeared to inhibit the enzyme activity of prothrombinase, an enzyme complex of factor Xa–factor Va–phospholipid–Ca^{2+}, which is responsible for prothrombin activation (Mann et al., 1981). We then tested the inhibitory effect of PAP on the activation of prothrombin by a reconstituted prothrombinase. The prothrombinase was reconstituted by 1.1 nM factor Xa, 9.5 nM factor Va, 0.1 μg of phospholipid, and 5 mM $CaCl_2$, and it was incubated with 8.9 μM prothrombin in the presence or absence of PAP. In order to ensure detection of any effect of PAP on phospholipid and factor Va, the concentrations of phospholipid and factor Va were chosen to be limiting for the prothrombinase activity. The prothrombinase activity was measured by assaying the amidolytic activity of thrombin that was generated in the reactions. Initial rates of thrombin formation decreased proportionally with the increasing amounts of PAP (Figure 7A). The prothrombinase activity was completely inhibited by 0.5 μg of PAP. When factor Va was omitted from the reaction mixture, the activation rate of prothrombin was 33-fold less than that observed with the

```
                    60                  70                  80                  90                 100
PAP           K G L G T D E E S I L T L L T S R S N A Q R Q E I S A A F K T L F G R D L L D D L K S E L
Lipocortin I  M V K G V D E A T I I D I L T K R N N A Q R Q Q I K A A Y L Q E T G K P L D E T L K K A L
Lipocortin II K T K G V D E V T I V N I L T N R S N A Q R Q D I A F A Y Q R R T K K E L A S A L K S A L

                   310                 320                 330                 340
PAP           V S R S E I D L F N I R K E F R K N F A T S L Y   I K G D T S G D Y K K A L L L L C G E D D
Lipocortin I  V S R S E I D M N D I K A F Y Q K M Y G I S L C Q A I L D E T K G D Y E K I L V A L C G G N
Lipocortin II V S R S E V D M L K I R S E F K R K Y G K S L Y Y Y I Q Q D T K G D Y Q K A L L Y L C G G D D
```

FIGURE 5: Sequence alignment of PAP fragments with lipocortin I and II. Residue numbers are taken from the lipocortin I sequence. The identical residues in all three proteins are boxed.

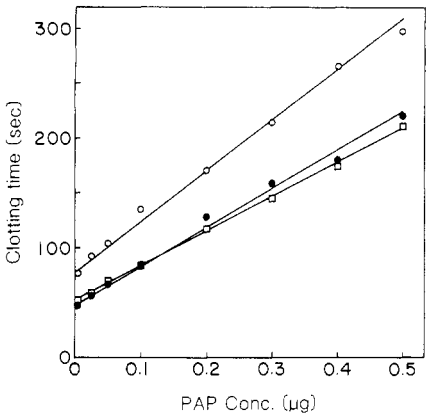

FIGURE 6: Anticoagulant activities of PAP. The anticoagulant activities of PAP were determined by kaolin-induced, thromboplastin-induced and factor Xa induced clotting times as described under Experimental Procedures. Inhibitory activities of PAP on kaolin-induced clotting time (○), thromboplastin-induced clotting time (□), and factor Xa induced clotting time (●).

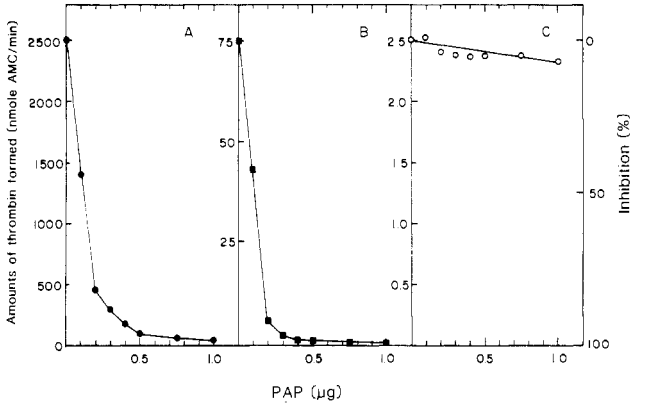

FIGURE 7: Effect of PAP on prothrombinase activity. Prothrombinase was composed of 1.1 nM factor Xa, 9.5 nM factor Va, 0.1 μg of phospholipid in 80 μL of buffer A containing BSA (0.1 mg/mL), and 5 mM $CaCl_2$. Prothrombinase was incubated with 8.9 μM prothrombin in the presence or absence of PAP. The prothrombinase activity was assayed by measuring thrombin activity with Boc-Val-Pro-Arg-MCA. (A) Effects of PAP on the complete prothrombinase (●); (B) reaction without factor Va (■); (C) reaction without phospholipid (○).

complete prothrombinase. The addition of PAP (0.3 μg) completely inhibited the thrombin formation (Figure 7B). When the prothrombin activation was conducted in the absence of phospholipid, the initial rate of the thrombin formation was far lower (1000-fold) than that obtained with the complete prothrombinase. In this reaction, the effect of PAP was not significant (Figure 7C), indicating that PAP did not affect the functional activity of either factor Va or factor Xa. These results strongly suggested that PAP inhibited the prothrombinase activity by interacting with phospholipid vesicles. Assuming the molecular weight of phospholipid is 700, it is calculated from the above experiments that one molecule of PAP blocks 17 molecules of PS/PC or 3.4 molecules of PS. Considering the random orientation (inside/outside) of PS in the reconstituted lipid membrane, 1 mol of PAP may block 1.7 mol of surface-exposed PS.

Binding of PAP to Phospholipid Vesicles. The binding capability of PAP to phospholipid was studied. Phospholipid vesicles were prepared by briefly sonicating a mixture of 20% PS and 80% PC. The ratio of PS to PC was chosen according to Nelsestuen and Broderius (1977), who showed that 15–20% of PS in PC had the maximum binding affinity to factor X and prothrombin.

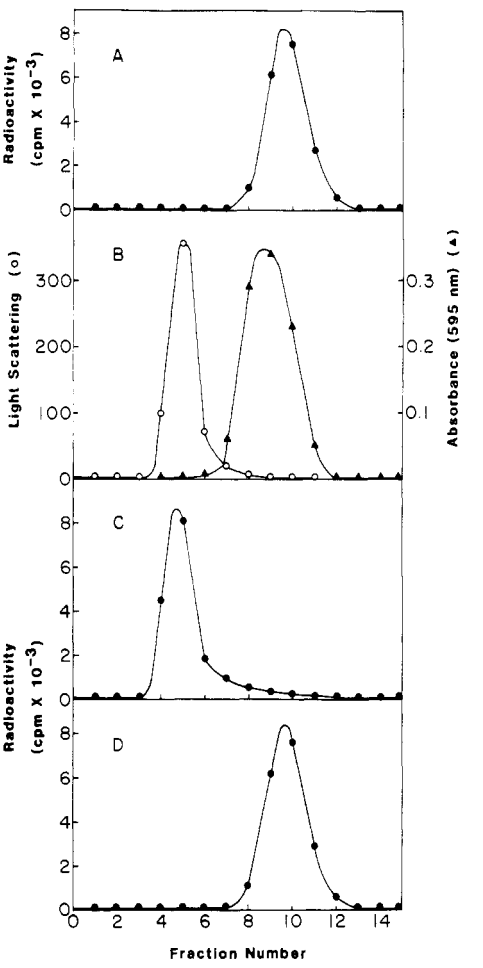

FIGURE 8: Binding of PAP to phospholipid vesicles. The complete binding mixture contained 0.1 mg of phospholipid vesicles, 5 μg of [^{3}H]PAP (total 2.5 × 10^5 cpm), and 0.1 mg of BSA in 210 μL of Tris-HCl buffer, pH 7.9, containing 1 mM $CaCl_2$ and 50 mM NaCl. The binding mixtures were then applied to a column (0.7 × 14 cm) of Sepharose 4B. Elution was performed with 50 mM Tris-HCl, pH 7.9, containing 50 mM NaCl at a flow rate of 10 mL/h. Fractions of 0.5 mL were collected. (A) Without phospholipid vesicles; (B) without PAP; (C) complete; (D) $CaCl_2$ replaced by EDTA in the binding mixture and $CaCl_2$ deleted from the buffer.

Binding experiments were performed with gel filtration on Sepharose 4B column in the presence of BSA and Ca^{2+}. When phospholipid was not present in the reaction, [^{3}H]PAP was detected in fractions 8–12 (Figure 8A). Phospholipid vesicle with a large molecular mass appeared in the void volume fractions (4–6). BSA, which did not bind to lipid vesicles, appeared in retarded fractions (Figure 8B). When PAP and phospholipid were both present, [^{3}H]PAP was detected in the void volume, demonstrating the binding of PAP to phospholipid vesicles (Figure 8C). When $CaCl_2$ was replaced by EDTA, PAP did not bind to phospholipid and it appeared in the same fractions as seen in the control experiment (Figure 8D). These experiments showed that binding of PAP to phospholipid was specific and required the presence of Ca^{2+} ion for its binding.

Since PAP inhibited the activity of prothrombinase (Figure 7), the interaction of PAP with either factor Xa, factor Va, or prothrombin was also possible. The binding of PAP to factor Xa, factor Va, and prothrombin was tested. [^{3}H]PAP was mixed with either factor Xa, factor Va, or prothrombin in the presence of 5 mM $CaCl_2$, and binding was tested by a gel filtration column as described under Experimental Procedures. In these experiments, [^{3}H]PAP was eluted in the same fraction as observed in the control experiment, where [^{3}H]PAP was applied alone to the column. These results

excluded the possibility of strong or irreversible binding of PAP to these factors. Although weak binding of PAP to factor Xa and factor Va is not ruled out, the results of these experiments are consistent with the observation that PAP does not inhibit prothrombinase activity in the absence of phospholipid (Figure 7C). Thus, it is not likely that PAP interacts with factor Xa or factor Va.

Discussion

The inhibition mechanism of PAP was found to interrupt prothrombinase activity by binding to one of its constituents, phospholipid, in a Ca^{2+}-dependent manner. These results suggest that PAP competes with factor Xa for binding to phospholipid. In this study, we demonstrated by gel filtration that PAP bound to phospholipid vesicles, while no binding to factor Xa or factor Va was observed. This technique, however, is not suitable for a quantitative study of competitive binding of PAP with factor Xa. Our preliminary study using fluorescence polarization and fluorescence quenching techniques showed that PAP has a strong binding affinity (K_d < 10 nM) for phospholipid vesicles containing phosphatidylserine. Furthermore, the binding is competitive with factor X or factor Xa (Tait et al., unpublished results). Since PAP interacts directly with phospholipid rather than factor Xa, other activation steps in the coagulation cascade, in which phospholipid is involved, are probably affected by PAP. These reactions are the activation of factor X by a complex of factor IXa–factor VIIIa–phospholipid–Ca^{2+} and the activations of factor X and factor IX by a tissue factor–factor VIIa–Ca^{2+} complex. The inhibition of tissue factor/factor VIIa activity by high-density lipoprotein has been reported (Carson, 1981; Kondo & Kisiel, 1986). Recently, apolipoprotein A-II, which was purified from plasma by dissociating lipid components by hydrophobic chromatographic media, was shown to inhibit the tissue factor/factor VIIa activity (Carson, 1987). The author reported that apolipoprotein prevented the appropriate association of tissue factor with factor VIIa. Thus, lipoprotein that circulates in blood as a complex with lipid seems to exchange the lipid partner, when it encounters with tissue factor–factor VIIa complex. The inhibition mechanism of PAP may be similar to that proposed for apolipoprotein A-II.

Reutelingsperger et al. (1985) reported the isolation of a novel inhibitor from arteries of human umbilical cord. This inhibitor inhibited prothrombin activation by prothrombinase. However, it did not suppress the prothrombin activation if phospholipid was deleted from the prothrombinase complex. Although direct evidence for the binding of this inhibitor to phospholipid has not been provided, the aurthors proposed that the inhibition mechanism of this inhibitor was a competition with factor Xa for binding to phospholipid. This protein is very similar to PAP as to the mode of inhibition. The molecular weight of this inhibitor is 32 000, which is slightly smaller than PAP. With the limited chemical characterization of this protein, presently it is difficult to determine if this inhibitor is identical with PAP. There are other anticoagulant proteins in the soluble fraction of placenta. We have found another inhibitory activity different from PAP in Sephadex G-75 fractions. The nonadsorbed fraction of the DEAE-Sephadex column also contains unidentified anticoagulant activities (possibly lipocortins). Thus, it is possible that the inhibitor that was isolated from the arteries of umbilical cord is the same as one of these proteins.

Determination of the amino acid sequence of PAP is in progress in our laboratory. The N-terminal residue of PAP is probably blocked, since amino acid sequence analysis of the intact protein failed to detect any sequence. The sequence analysis of the cyanogen bromide fragments revealed that PAP was a new member of the lipocortin or calpactin family. The sequences of several cyanogen bromide fragments of PAP align with the sequences of lipocortin I and II (Huang et al., 1986; Saris et al., 1986; Wallner et al., 1986) with over 50% identity. These sequences include the putative binding sites of lipocortins to phospholipid vesicles (Weber & Johnsson, 1986; Geisow, 1986). Lipocortins inhibit phospholipase A_2 activity by Ca^{2+}-dependent binding to its substrate, phospholipid (Glenney, 1986; Davidson et at., 1987). The inhibition of phospholipase A_2 blocks the release of arachidonic acid and hence may diminish the production of prostaglandins. Synthesis of lipocortins is stimulated by glucocorticoids, which are important antiinflammatory agents (Flower & Blackwell, 1979; Blackwell et al., 1980). In preliminary experiments, we have found that PAP also inhibits phospholipase A_2 activity. Although it has not been reported, anticoagulant activities of lipocortins can be anticipated, because lipocortins bind phospholipid and have sequences homologous to PAP. However, the strength of the anticoagulant activity of lipocortins, if any, may be different depending on their binding preference for lipid components.

At the present time, the physiological role and origin of PAP are not known. PAP may originate from the endothelium of placenta, because we have detected a PAP-like anticoagulant activity in bovine aortic endothelial cells. This activity and PAP behaved similarly in the purification up to the gel filtration step. If PAP antibody recognizes the antigen in the endothelial cells, it will be interesting to see whether PAP localizes on the surface or inside the cells. Nevertheless, if PAP is present in endothelial cells, it may play an antithrombotic role. Heimark and Schwartz (1983) and Stern and co-workers (1983) discovered the binding of factor IX and factor X to the surface of endothelial cells. The latter group demonstrated fibrin formation on the endothelial surface, when the endotoxin-treated cells were incubated with factor VIIa, factor VIII, factor X, factor IX, prothrombin, and fibrinogen (Stern et al., 1985), suggesting a physiological function for endothelial cells in thrombus formation. PAP may bind phospholipid components at injured sites, before coagulation factors come in contact with lipid components and initiate thrombosis.

Acknowledgments

We are grateful to Dr. Earl W. Davie for his support and encouragement throughout this work. We also thank Drs. Jonathan F. Tait and William H. Kane for their valuable suggestions.

Registry No. Thromboplastin, 9035-58-9; factor Xa, 9002-05-5; thrombin, 9002-04-4; prothrombinase, 72162-96-0; prothrombin, 9001-26-7; factor Va, 65522-14-7.

References

Bevilacqua, M. P., Pober, J. S., Majeau, G. R., Fiers, W., Cotran, R. S., & Gimbrone, M. a., Jr. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 4533–4537.

Blackwell, G. J., Carnuccio, R., Di Rosa, M., Flower, R. J., Parente, L., & Persico, P. (1980) *Nature (London) 287*, 147–149.

Bradfor, M. M. (1976) *Anal. Biochem. 72*, 248–254.

Carson, S. D. (1981) *FEBS Lett. 132*, 37–40.

Carson, S. D. (1987) *J. Biol. Chem. 262*, 718–721.

Crestfield, A. M., Moore, S., & Stein, W. H. (1963) *J. Biol. Chem. 238*, 622–627.

Davidson, F. F., Dennis, E. A., Powell, M., & Glenney, J. R., Jr. (1987) *J. Biol. Chem. 262*, 1698–1705.

Ellman, G. L. (1959) *Arch. Biochem. Biophys. 82*, 70–77.

Esmon, N. L., Owen, W. G., & Esmon, C. T. (1982) *J. Biol. Chem. 257*, 859–864.
Florey, L. (1966) *Br. Med. J. 2*, 487–490.
Flower, R. J., & Blackwell, G. J. (1979) *Nature (London) 278*, 456–459.
Fraker, P. J., & Speck, J. C. (1978) *Biochem. Biophys. Res. Commun. 80*, 849–857.
Fujikawa, K., Legaz, M. E., & Davie, E. W. (1972) *Biochemistry 11*, 4892–4899.
Geisow, M. J. (1986) *FEBS Lett. 203*, 99–103.
Glenney, J. (1986) *J. Biol. Chem. 261*, 7247–7252.
Glossman, H., & Neville, D. M., Jr. (1971) *J. Biol. Chem. 246*, 6339–6346.
Harboe, N., & Ingild, A. (1973) *Scand. J. Immunol. 2 (Suppl. 1)*, 161–164.
Heimark, R. L., & Schwartz, S. M. (1983) *Biochem. Biophys Res. Commun. 111*, 723–731.
Huang, K.-S., Wallner, B. P., Mattaliano, R. J., Tizard, R., Burne, C., Frey, A., Hession, C., McGray, P., Sinclair, L. K., Chow, E. P., Browning, J. L., Ramachandran, K. L., Tang, J., Smart, J. E., & Pepinsky, R. B. (1986) *Cell (Cambridge, Mass.) 46*, 191–199.
Kisiel, W., Canfield, W. M., Ericsson, L. H., & Davie, E. W. (1977) *Biochemistry 16*, 5824–5831.
Kondo, S., & Kisiel, W. (1986) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 45*, 1073.
Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.
Lundblad, R. L., Kingdon, H. S., & Mann, K. G. (1976) *Methods Enzymol. 45*, 156–176.
Mann, K. G., Elion, J., Butkowski, R. J., Downing, M., & Nesheim, M. E. (1981) *Methods Enzymol. 80*, 286–302.
Marcum, J. A., McKenney, J. B., & Rosenberg, R. D. (1984) *J. Clin. Invest. 74*, 341–350.
Mason, R. G., Sharp, D., Chuang, H. Y. K., & Mohammad, S. F. (1977) *Arch. Pathol. Lab. Med. 101*, 61–64.
Maynard, J. R., Dreyer, B. E., Stemmerman, M. B., & Pitlick, F. A. (1977) *Blood 50*, 387–396.
McMullen, B. A., & Fujikawa, K. (1985) *J. Biol. Chem. 260*, 5328–5341.
Means, G. E., & Feeney, R. E. (1968) *Biochemistry 7*, 2192–2201.
Miletich, J. P., Broze, G. J., Jr., & Majerus, P. W. (1981) *Methods Enzymol. 80*, 221–228.
Morita, T., Kato, H., Iwanaga, S., Takada, K., Kimura, T., & Sakakibara, S. (1977) *J. Biochem. (Tokyo) 82*, 1495–1498.
Nawroth, P. P., Handley, D. A., Esmon, C. T., & Stern, D. M. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 3460–3464.
Nelsestuen, G. L. & Broderius, M. (1977) *Biochemistry 16*, 4172–4177.
Owen, W. G., & Esmon, C. T. (1981) *J. Biol. Chem. 256*, 5532–5535.
Reutelingsperger, C. P. M., Hornstra, G., & Hemker, H. C. (1985) *Eur. J. Biochem. 151*, 625–629.
Salem, H. H., Maruyama, I., Ishii, H., & Majerus, P. W. (1984) *J. Biol. Chem. 259*, 12246–12251.
Saris, C. J. M., Tack, B. F., Kristensen, T., Glenney, J. R., Jr., & Hunter, T. (1986) *Cell (Cambridge, Mass.) 46*, 201–212.
Seegers, W. H. (1964) in *Blood Coagulation, Hemorrhage and Thrombosis* (Tocantins, L. M., & Kazal, L. A., Eds.) pp 181–187, Grune & Stratton, New York.
Stern, D. M., Drillings, M., Nossel, H. L., Hurlet-Jensen, A., LaGamma, K. S., & Owen, J. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 4119–4123.
Stern, D., Nawroth, P., Handley, D., & Kisiel, W. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 2523–2527.
Towbin, H., Staehelin, T., & Gordon, J. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 4350–4354.
Vehar, G. A., & Davie, E. W. (1980) *Biochemistry 19*, 401–410.
Wallner, B. P., Mattaliano, R. J., Hession, C., Cate, R. L., Tizard, R., Sinclair, L. K., Foeller, C., Chow, E. P., Browning, J. L., Ramachandran, K. L., & Pepinsky, R. B. (1986) *Nature (London) 320*, 77–81.
Weber, K., & Osborn, M. (1969) *J. Biol. Chem. 244*, 4406–4412.
Weber K., & Johnsson, N. (1986) *FEBS Lett. 203*, 95–98.