& Vriend, G. (1985) *Nature (London) 317*, 145-153.
Sanger, F., Nicklen, S., & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5463-5467.
Schoner, R. G., Ellis, L. F., & Schoner, B. E. (1985) *Bio/Technology 3*, 151-154.
Skern, T., Sommergruber, W., Blaas, D. Gruendler, P., Fraundorfer, F., Pieler, C., Fogg, I., & Kuechler, E. (1985) *Nucleic Acids Res. 13*, 2111-2126.
Stanway, G., Hughes, P. J., Mountford, R. C., Minor, P. D., & Almond, J. W. (1984) *Nucleic Acids Res. 12*, 7859-7875.
Stott, E. J., & Killington, R. A. (1972) *Annu. Rev. Microbiol. 26*, 503-524.
Tessier, L.-H., Sondermeyer, P., Faure, T., Dreyer, D., Benavente, A., Villeval, D., Courtney, M., & Lecocq, J.-P. (1984) *Nucleic Acids Res. 12*, 7663-7675.
Towbin, H., Staehelin, T., & Gordon, J. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 4350-4354.
Toyoda, H., Nicklin, M. J. H., Murray, M. G., Anderson, C. W., Dunn, J. J., Studier, F. W., & Wimmer, E. (1986) *Cell (Cambridge, Mass.) 45*, 761-770.

Placental Anticoagulant Proteins: Isolation and Comparative Characterization of Four Members of the Lipocortin Family†

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ABSTRACT: Previously we isolated and characterized a placental anticoagulant protein (PAP or PAP-I), which is a Ca^{2+}-dependent phospholipid binding protein [Funakoshi et al. (1987) *Biochemistry 26*, 5572] and a member of the lipocortin family [Funakoshi et al. (1987) *Biochemistry 26*, 8087]. In this study, three additional anticoagulant proteins (PAP-II, PAP-III, and PAP-IV) were simultaneously isolated from human placental homogenates prepared in the presence of 5 mM ethylenediaminetetraacetic acid. The isoelectric points of PAP-I, PAP-II, PAP-III, and PAP-IV were 4.8, 6.1, 5.9, and 8.1, respectively, and their apparent molecular weights were 32 000, 33 000, 34 000, and 34 500, respectively. Amino acid sequences of cyanogen bromide fragments of these proteins showed that PAP-III was a previously unrecognized member of the lipocortin family, while PAP-II was probably the human homologue of porcine protein II and PAP-IV was a derivative of lipocortin II truncated near the amino terminus. Comparative studies showed that all four proteins inhibited blood clotting and phospholipase A_2 activity with potencies consistent with their measured relative affinities for anionic phospholipid vesicles. However, PAP-IV bound to phospholipid vesicles approximately 160-fold more weakly than PAP-I, while PAP-II and PAP-III bound only 2-fold and 3-fold more weakly. These results increase to six the number of lipocortin-like proteins known to exist in human placenta. The observed differences in phospholipid binding may indicate functional differences among the members of the lipocortin family despite their considerable structural similarities.

Phospholipid surfaces are essential components in several key reactions of the coagulation cascade (Mann, 1984; Zwaal et al., 1986). As such, they represent a potential regulatory point for physiological or pharmacological control of blood coagulation. Recently, we described the identification and purification of a candidate anticoagulant protein from human placenta termed placental anticoagulant protein (PAP or PAP-I)[1] (Funakoshi et al., 1987a). This protein inhibits the extrinsic and intrinsic pathways of blood coagulation and binds specifically to anionic phospholipid surfaces in the presence of Ca^{2+} (Funakoshi et al., 1987a; Kondo et al., 1987). Iwasaki et al. (1987) have also purified and characterized the same anticoagulant protein (termed inhibitor of blood coagulation) from human placenta, and Reutelingsperger et al. (1985) have isolated a similar protein (vascular anticoagulant) from human umbilical cord arteries. This protein has also been purified from human placenta during studies of potential substrates of the epidermal growth factor receptor/kinase, and it has been called endonexin II (Haigler et al., 1987; Schlaepfer et al., 1987).

Protein and cDNA sequence data (Funakoshi et al., 1987a,b; Iwasaki et al., 1987; Schlaepfer et al., 1987) show that PAP-I is a member of a recently described family of Ca^{2+}-dependent phospholipid binding proteins variously termed lipocortins (Wallner et al., 1986; Huang et al., 1986), calpactins (Saris et al., 1986; Kristensen et al., 1986; Glenney, 1986a), proteins I, II, and III (Gerke & Weber, 1984; Shadle et al., 1985), calelectrins (Walker et al., 1983; Sudhof et al., 1984, 1988), annexins (Geisow, 1986), p35 and p36 (Fava & Cohen, 1984; De et al., 1986; Gerke & Weber, 1984; Glenney & Tack, 1985), chromobindins (Creutz et al., 1987), or calcimedins (Moore & Dedman, 1982; Smith & Dedman, 1986). The physiological roles of these proteins are presently unknown. They have been proposed to participate in membrane fusion and exocytosis (Creutz, 1981; Geisow & Burgoyne, 1982; Sudhof et al., 1982), cytoskeleton–membrane linkage (Walker,

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[1] Abbreviations: C_6-NBD-PC, 1-palmitoyl-2-[[*N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]caproyl]phosphatidylcholine; EDTA, ethylenediaminetetraacetic acid; FITC, fluorescein-5-isothiocyanate; FPLC, fast protein liquid chromatography; HEPES, *N*-(2-hydroxyethyl)-piperazine-*N'*-2-ethanesulfonic acid; HPLC, high-performance liquid chromatography; IC_{50}, concentration of protein causing 50% inhibition of binding; PAP, placental anticoagulant protein; PC, phosphatidylcholine; PS, phosphatidylserine; SDS, sodium dodecyl sulfate.

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1982; Gerke & Weber, 1984; Glenney, 1986a), control of inflammation through regulation of phospholipase activity (Flower & Blackwell, 1979; Blackwell et al., 1980; Hirata et al., 1980; Wallner et al., 1986), and regulation of blood coagulation (Funakoshi et al., 1987a,b; Kondo et al., 1987; Iwasaki et al., 1987). So far, complete sequences are available for five proteins in this family: (1) human lipocortin I (Wallner et al., 1986) and rat lipocortin I (Tamaki et al., 1987); (2) human lipocortin II (Huang et al., 1986), murine calpactin I heavy chain (Saris et al., 1986), and bovine calpactin I heavy chain (Kristensen et al., 1986); (3) human PAP-I (Funakoshi et al., 1987b)/human inhibitor of blood coagulation (Iwasaki et al., 1987); (4) porcine protein II (Weber et al., 1987); (5) human 67-kDa calelectrin or p68 (Sudhof et al., 1988; Crompton et al., 1988).

During the purification of PAP-I, other anticoagulant activities were observed in the soluble fraction of placental homogenates (Funakoshi et al., 1987a; Iwasaki et al., 1987). Since PAP-I, lipocortin I, and lipocortin II are known to be present in placenta, it is necessary to establish whether the observed anticoagulant activities represent these known proteins, their precursors or proteolytically cleaved products, other proteins related to PAP-I or the lipocortins, or possibly a new type of anticoagulant. It is also necessary to study the potency of these inhibitors to evaluate their potential significance in blood coagulation and their potential utility as pharmacological anticoagulants.

In this study, we have purified and characterized three additional anticoagulant proteins from human placenta, which we have termed PAP-II, PAP-III, and PAP-IV. PAP-III is a previously unrecognized member of the lipocortin family; PAP-II is probably the human homologue of protein II from porcine intestinal mucosa (Weber et al., 1987) or endonexin from bovine liver (Geisow et al., 1986); PAP-IV is a truncated form of lipocortin II, which is cleaved near the amino terminus. We have also performed comparative studies of the potencies of these proteins in binding to phospholipid and as inhibitors of blood clotting and phospholipase A_2 activity. While PAP-I, PAP-II, and PAP-III have similar potencies, PAP-IV/lipocortin II is far less potent than the other three.

Experimental Procedures

Materials. Materials were from the following sources: ovalbumin, grade V, porcine pancreatic phospholipase A_2, and HEPES (Sigma); diheptanoyl-PC, 1-palmitoyl-2-oleoyl-PC, 1-palmitoyl-2-oleoyl-PS (disodium salt), and C_6-NBD-PC (Avanti, Birmingham, AL); isoelectric focusing gels (Phast Gel IEF 3-9) and protein isoelectric point and molecular weight standards (Pharmacia).

Purification of PAP-II, PAP-III, and PAP-IV. The starting material was a DEAE-Sepharose effluent fraction prepared as described previously (Funakoshi et al., 1987a). This fraction (2 L) was dialyzed overnight against 12 L of 50 mM sodium acetate buffer, pH 5.2, containing 0.5 mM EDTA, with one change of buffer. The dialysand was then mixed with 350 mL of CM-Sephadex C-50 previously equilibrated with the same buffer and stirred for 2 h. After the supernatant was discarded, the slurry was poured into a plastic column (4.5 × 30 cm), and the column was washed with 2 L of the acetate buffer. Adsorbed proteins were then eluted with a linear gradient system composed of 1.0 L of 0 M and 1.0 L of 0.5 M NaCl in the same buffer, and 10-mL fractions were collected. Every fifth fraction was then assayed for anticoagulant activity, which emerged in two broad but discrete peaks at salt concentrations of approximately 0.20–0.30 and 0.35–0.50 M. The fractions of the first and second peaks were pooled separately, and proteins in the pooled fractions were precipitated by adding ammonium sulfate to 80% saturation. The precipitates were dissolved in a minimum volume of 50 mM Tris-HCl buffer, pH 7.9, containing 0.2 M NaCl, 1 mM EDTA, and 0.5 mM benzamidine and dialyzed briefly against the same buffer.

The first and second peaks from the CM-Sephadex column were then separately subjected to gel filtration as follows. The dialyzed samples (~70 mL) were applied to a column (5 × 150 cm) of Sephadex G-75 superfine equilibrated with 50 mM Tris-HCl buffer, pH 7.9, containing 0.2 M NaCl, 1 mM EDTA, and 0.5 mM benzamidine. The column was eluted with the same Tris-HCl buffer. Anticoagulant activity appeared at the descending edge of a major protein peak. The active fractions were pooled and concentrated by ammonium sulfate precipitation as described above. The concentrated sample (~50 mL) was then reapplied to the same column. Anticoagulant activity appeared with a small peak at the descending edge of the major protein peak. The active fractions from the second cycle of gel filtration were pooled and dialyzed against 4 L of 25 mM sodium acetate buffer, pH 5.2, containing 0.5 mM EDTA, with one change of buffer.

The dialyzed samples were divided into four to five aliquots, and each was applied to a Mono S column (0.5 × 5 cm) connected to a Pharmacia FPLC system. Proteins were eluted at 1 mL/min by a linear NaCl gradient in 25 mM sodium acetate buffer, pH 5.2, containing 0.5 mM EDTA. The effluent was monitored by the absorbance at 280 nm, and 0.5-mL fractions were collected. Every other fraction was assayed for anticoagulant activity and inhibition of phospholipase A_2. The purified anticoagulant proteins were pooled separately, dialyzed against 50 mM Tris-HCl buffer, pH 7.9, containing 0.5 mM EDTA, and stored frozen until used.

Preparation of Cyanogen Bromide Fragments and Sequence Analysis. Proteins (2–3 mg) were carboxymethylated according to Crestfield et al. (1963), and excess reagents were removed by dialysis against 5% HCOOH followed by lyophilization. Salt-free carboxymethylated proteins were cleaved at room temperature for 24 h with 1 mL of 2% cyanogen bromide in 70% HCOOH. The resulting fragments were sized into four or five subfractions by a gel filtration column of Sephadex G-50 superfine (1.5 × 95 cm) eluted with 5% HCOOH. Fragments in each subfraction were further separated by HPLC on an Altex C3 reversed-phase column (0.46 × 7.5 cm) as described previously (Fujikawa & McMullen, 1983). Sequence analysis was performed on a Beckman sequenator, Model 890C, by the method of Edman and Begg (1967). Phenylthiohydantoin-amino acids were identified by two complementary HPLC systems (Bridgen et al., 1976; Ericsson et al., 1977).

Electrophoresis and Determination of Protein Concentration. SDS–polyacrylamide gel electrophoresis was performed according to Weber and Osborn (1969). Protein concentrations were determined by the method of Bradford (1976) using bovine serum albumin as a standard. Isoelectric focusing was performed at 15 °C and 2000 V for 400 V-h on the Pharmacia PhastSystem (Pharmacia); proteins were visualized by Coomassie blue staining. The following standards were used (p*I* values given in parentheses): amyloglucosidase (3.50), soybean trypsin inhibitor (4.55), β-lactoglobulin (5.20), bovine carbonic anhydrase B (5.85), human carbonic anhydrase B (6.55), horse myoglobin, acidic band (6.85), horse myoglobin, basic band (7.35), lentil lectin, acidic band (8.15), lentil lectin, middle band (8.45), lentil lectin, basic band (8.65), and trypsinogen (9.30).

Fluorescence Polarization Immunoassay. FITC-PAP-I was prepared as described elsewhere.[2] Rabbit polyclonal IgG against PAP-I was prepared and affinity-purified as described (Funakoshi et al., 1987a). Assays were performed in a buffer consisting of 120 mM NaCl, 2.7 mM KCl, 10 mM sodium phosphate, pH 7.4, 1 mM EDTA, and 0.1% (v/v) Triton X-100. Cuvettes were prepared with 1.6 nM FITC-PAP-I, 14 nM affinity-purified IgG, and a varying amount of unlabeled competitor protein. After an 8-min incubation at 34 °C, fluorescence polarization was measured with a TDx Analyzer (Abbott Laboratories), and net fluorescence polarization was calculated as described (Tait et al., 1986). The percentage of FITC-PAP-I bound compared to a control without competitor protein was then calculated from the observed net polarization change relative to the net polarization change observed in the absence of competitor protein.

Preparation of Phospholipid Vesicles. Small unilamellar phospholipid vesicles were prepared according to Gabriel and Roberts (1984). In this procedure, addition of approximately 20 mol % diheptanoyl-PC to long-chain phospholipids leads to the spontaneous formation of unilamellar vesicles. Aliquots of phospholipid stock solutions in chloroform were mixed to yield the desired molar ratios, and the chloroform was removed by evaporation with nitrogen. The phospholipids were then dissolved in 0.05 M NaHEPES, pH 7.4, 0.10 M NaCl, and 3 mM NaN_3 by sonication for 3 min on ice, followed by overnight equilibration at 4 °C. The phospholipid concentration was determined by phosphate analysis (Chen et al., 1956) after digestion of samples with 70% perchloric acid at 160 °C for 3 h. For binding studies and clotting assays, phospholipid vesicles (referred to as "PC/PS") consisted of diheptanoyl-PC/1-palmitoyl-2-oleoyl-PC/1-palmitoyl-2-oleoyl-PS in molar ratios of 20/60/20. For phospholipase A_2 assays, phospholipid vesicles (referred to as "C_6-NBD-PC/PS") consisted of C_6-NBD-PC/1-palmitoyl-2-oleoyl-PS in a molar ratio of 80/20. (Diheptanoyl-PC was omitted from these preparations because C_6-NBD-PC is much more water-soluble than 1-palmitoyl-2-oleoyl-PC and spontaneously forms micelles or small vesicles.)

Phospholipid Binding Assay. Binding of PAP-I, PAP-II, PAP-III, and PAP-IV to PC/PS vesicles was measured by a competition assay to be described fully elsewhere (Tait and Fujikawa, unpublished results). In this procedure, binding of FITC-PAP-I to PC/PS vesicles can be measured by fluorescence quenching. Briefly, unlabeled competitor protein was incubated at 25 °C with FITC-PAP-I (0.86 nM) and PC/PS (2.4 μM) for 2 min in a buffer consisting of 0.05 M Na-HEPES, pH 7.4, containing 0.1 M NaCl, 3 mM NaN_3, 1.2 mM $CaCl_2$, and 5 μg/mL ovalbumin. The percentage of FITC-PAP-I bound to vesicles compared to a control without competitor protein was then calculated from the observed fluorescence quenching divided by the quenching obtained in the absence of competitor protein. Fluorescence measurements were performed on an LS-5 fluorometer (Perkin-Elmer) with excitation at 495 ± 5 nm and emission monitored at 520 ± 20 nm.

Clotting Assay. Routine assays of column fractions were performed by the kaolin-activated partial thromboplastin time using rabbit brain cephalin as described previously (Funakoshi et al., 1987a). For studies of the purified proteins, synthetic phospholipids (PC/PS) were substituted for rabbit brain cephalin. Final assay conditions were as follows: 2.4 μM PC/PS, 6.6 mM $CaCl_2$, ionic strength 0.16, pH 7.4, temperature 37 °C. The control clotting time was approximately 100 s under these conditions.

Phospholipase A_2 Assay. Phospholipase A_2 activity was determined fluorometrically with the substrate C_6-NBD-PC. The fluorescence intensity increases greatly when the NBD fluorophore is released from self-quenching in C_6-NBD-PC vesicles by detergents or hydrolysis (Nichols & Pagano, 1981; Wittenauer et al., 1984). Reaction mixtures were prepared with 3.2 μM C_6-NBD-PC/PS and varying concentrations of competitor protein in the same buffer used for binding studies (see above). Following a 1-min preincubation at 25 °C, the base-line fluorescence was measured. Phospholipase A_2 was then added (21 nM final concentration) and the fluorescence measured again after a 1-min incubation at 25 °C. Percent activity was then calculated by dividing the observed fluorescence increase by the increase observed in the absence of competitor protein. Consumption of substrate was less than 5% during the period of the measurement. The excitation wavelength was 480 ± 15 nm and the emission wavelength 550 ± 20 nm.

Results

Isolation of PAP-II, PAP-III, and PAP-IV. The final purification of the three proteins was performed on a cation-exchange Mono S column connected to a Pharmacia FPLC system. The Mono S elution profile of material from the first peak of the CM-Sephadex column is shown in Figure 1A. There were two anticoagulant activities, which were associated with protein peaks eluting at NaCl concentrations of 0.06 and 0.08 M. Since PAP-I inhibits phospholipase A_2 (Haigler et al., 1987; Tait and Fujikawa, unpublished results), fractions were also assayed for inhibition of phospholipase A_2. Inhibition of phospholipase A_2 activity paralleled the anticoagulant effect. Ouchterlony immunoprecipitation showed that the protein in the first activity peak was the same protein as the previously isolated "PAP"; this protein is now renamed "PAP-I". [Most of the PAP-I in placental extracts adsorbs to the DEAE-Sepharose column; this material was purified from the DEAE-Sepharose eluate by the same steps of gel filtration and Mono S chromatography used in this study (Funakoshi et al., 1987a). However, a small portion of PAP-I passes through the DEAE-Sepharose column, accounting for the first peak in Figure 1A.] The protein associated with the second peak was named PAP-II (Figure 1A). Similarly, two other anticoagulant proteins were found in the Mono S elution profile of material from the second anticoagulant peak of the CM-Sephadex column (Figure 1B). These proteins adsorbed more tightly to the Mono S column than PAP-I and PAP-II and eluted at salt concentrations of 0.17 and 0.27 M. These two anticoagulant proteins are named PAP-III and PAP-IV. We obtained 3 mg of PAP-II, 1 mg of PAP-III, and 3 mg of PAP-IV from one placenta. If we assume that the recoveries of these proteins are similar during the purification, these numbers indicate that PAP-I, obtained in a yield of 20–25 mg/placenta, is by far the major protein of this family in human placenta.

Purity and Molecular Weights of PAP-I, PAP-II, PAP-III, and PAP-IV. PAP-I, PAP-II, PAP-III, and PAP-IV were all homogeneous on SDS–polyacrylamide gels performed under reducing conditions (Figure 2). The estimated molecular weights of PAP-I, PAP-II, PAP-III, and PAP-IV are 32 000, 33 000, 34 000, and 34 500, respectively. The presently estimated molecular weight of PAP-I is different from the previously determined value of 36 500 (Funakoshi et al., 1987a). The reason for this discrepancy is not known. Nevertheless, the molecular weight of PAP-I was found to be 35 847 from

[2] J. F. Tait and K. Fujikawa, unpublished data.

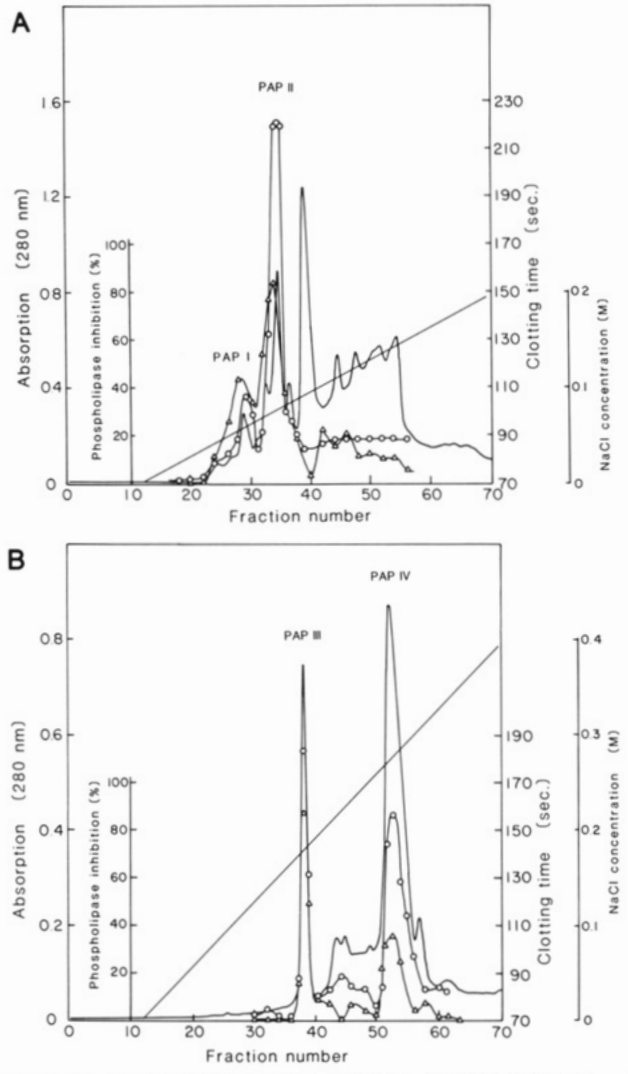

FIGURE 1: Final purification step of PAP-I, PAP-II, PAP-III, and PAP-IV by Mono S column chromatography. The pooled materials from the gel filtration column were prepared as described under Experimental Procedures and applied to a Mono S column connected to a Pharmacia FPLC system. The column was equilibrated with 25 mM sodium acetate buffer, pH 5.2, containing 0.5 mM EDTA, and elution was performed by a linear NaCl gradient. (A) Separation of PAP-I and PAP-II; (B) separation of PAP-III and PAP-IV. Absorption at 280 nm (line without symbols); anticoagulant activity (line with circles); inhibitory activity against phospholipase A_2 (line with triangles).

the completed sequence (Funakoshi et al., 1987b). Thus, the molecular weights of PAP-II, PAP-III, and PAP-IV could also be approximately 4000 larger than the values obtained by gel electrophoresis.

Other Chemical and Immunological Characterization. Isoelectric points were determined as 4.8 for PAP-I, 6.1 for PAP-II, 5.9 for PAP-III, and 8.1 for PAP-IV (Figure 3). A minor isoform of PAP-IV was observed, possibly due to proteolytic cleavages near the amino terminus (see below). Competitive immunoassay (Figure 4) demonstrated that PAP-II, PAP-III, and PAP-IV had virtually no cross-reactivity with a polyclonal antiserum against PAP-I. Free sulfhydryl groups were determined in the presence of 6 M guanidine according to Ellman (1959), and the following numbers were obtained: 4.2 for PAP-II, 3.1 for PAP-III, and 2.5 for PAP-IV. The value of 0.8 was previously obtained for PAP-I (Funakoshi et al., 1987a). None of these proteins was positive when polyacrylamide gels were stained by Schiff reagent (Glossmann & Neville, 1971), indicating that these proteins

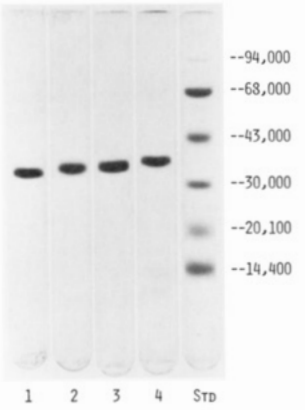

FIGURE 2: SDS–polyacrylamide gel electrophoresis of PAP-I, PAP-II, PAP-III, and PAP-IV. Reduced samples (5 μg) were applied to 7.5% acrylamide gels, and electrophoresis was carried out at 8 mA/tube for 3 h. Samples (from the left) were PAP-I (1), PAP-II (2), PAP-III (3), PAP-IV (4), and molecular weight standards (Std).

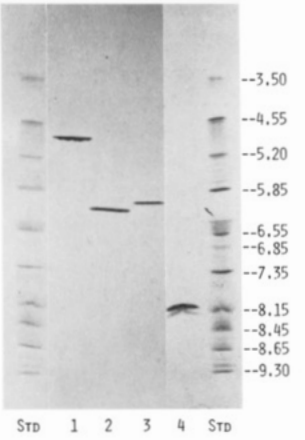

FIGURE 3: Isoelectric focusing of PAP-I, PAP-II, PAP-III, and PAP-IV. Approximately 0.5–1 μg of protein was applied to each lane; electrophoresis was then performed as described under Experimental Procedures. The isoelectric points of the standards are given at the right of the figure. Samples (from the left) were standards (Std), PAP-I (1), PAP-II (2), PAP-III (3), PAP-IV (4), and standards (Std).

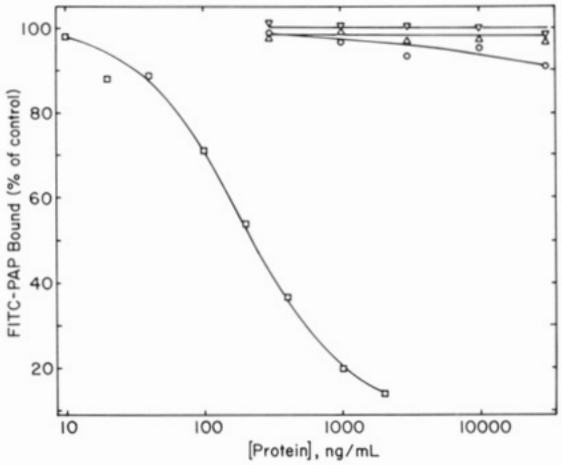

FIGURE 4: Immunological cross-reactivity of PAP-I, PAP-II, PAP-III, and PAP-IV with PAP-I antibody. Fluorescence polarization immunoassay was performed with varying amounts of unlabeled competitor protein as described under Experimental Procedures. Symbols: PAP-I (squares); PAP-II (circles); PAP-III (triangles); PAP-IV (inverted triangles).

are not conjugated with carbohydrate.

Partial Amino Acid Sequence and Alignment with Proteins of the Lipocortin Family. Since the amino terminus of PAP-I is blocked by an acetyl group (Funakoshi et al., 1987b; Iwasaki

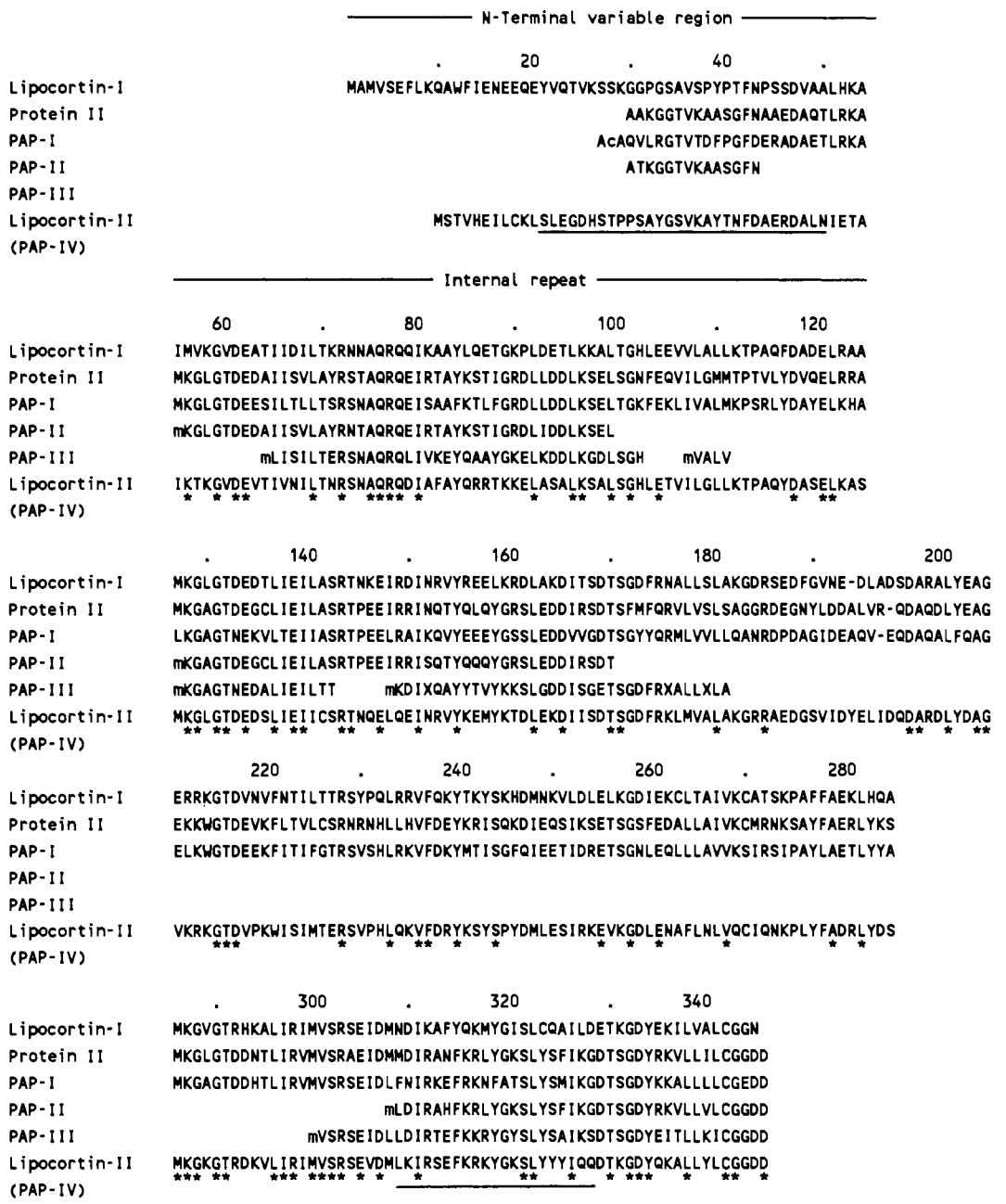

FIGURE 5: Amino acid sequences of cyanogen bromide fragments of PAP-II, PAP-III, and PAP-IV, and their alignments with PAP-I and lipocortins. Asterisks indicate conserved residues in the internal repeats. "m" represents expected methionine residues. "Ac" refers to an acetyl group. "X" indicates an unidentified residue. Gaps (–) were placed to improve alignments. The two segments underlined in the lipocortin II sequence were sequenced with the fragments obtained from PAP-IV. Sources of complete sequences were as follows: human lipocortin I, Wallner et al. (1986); human lipocortin II, Huang et al. (1986); human PAP-I, Funakoshi et al. (1987b); porcine protein II, Weber et al. (1987).

et al., 1987), intact preparations of PAP-II, PAP-III, and PAP-IV were first examined for the nature of their amino-terminal residues. Sequence was obtained in reasonably good yield only for PAP-IV, suggesting that the amino-terminal residues of PAP-II and PAP-III are probably blocked. The carboxymethylated proteins were then cleaved with cyanogen bromide, and the resulting fragments were separated by a combination of gel filtration and reversed-phase HPLC as described under Experimental Procedures. Several fragments from each protein were then subjected to sequence analysis.

Four fragments from PAP-II were sequenced, and 143 residues were identified. These fragments gave sequences homologous to the complete sequences of proteins in the lipocortin family, and they could be aligned with those proteins in four distinct regions (Figure 5). These fragments have 95% sequence identity with porcine protein II, indicating that PAP-II is likely to be the human homologue of this protein.

Similarly, 5 fragments of PAP-III were sequenced, and 139 residues were identified. These aligned with 52% identity with PAP-I, 57% with porcine protein II, 54% with lipocortin I, and 54% with lipocortin II (Figure 5). These results clearly show that this protein is a newly described member of the lipocortin family; its sequence has not been previously recognized in the proteins isolated from human placenta or other sources. Fragments were identified corresponding to three of the four internal repeats characteristic of the lipocortins, indicating that PAP-III is likely to have the same structural motifs as the other lipocortins.

The amino-terminal sequence of intact PAP-IV was identical with the lipocortin II sequence (Huang et al., 1986) starting at residue 12, indicating posttranslational proteolytic cleavage occurred between Leu-11 and Ser-12. Another

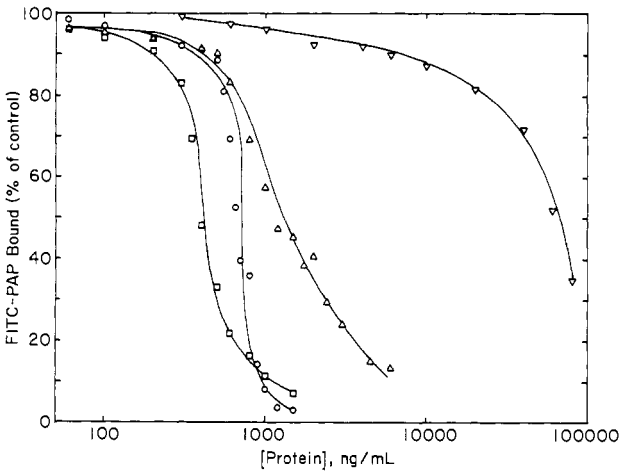

FIGURE 6: Binding of PAP-I, PAP-II, PAP-III, and PAP-IV to PC/PS vesicles. Binding assays were performed as described under Experimental Procedures by measuring the ability of each unlabeled protein to compete with FITC-PAP-I for binding to PC/PS vesicles. The final concentration of PC/PS (80/20) was 2.4 μM. Symbols: PAP-I (squares); PAP-II (circles); PAP-III (triangles); PAP-IV (inverted triangles).

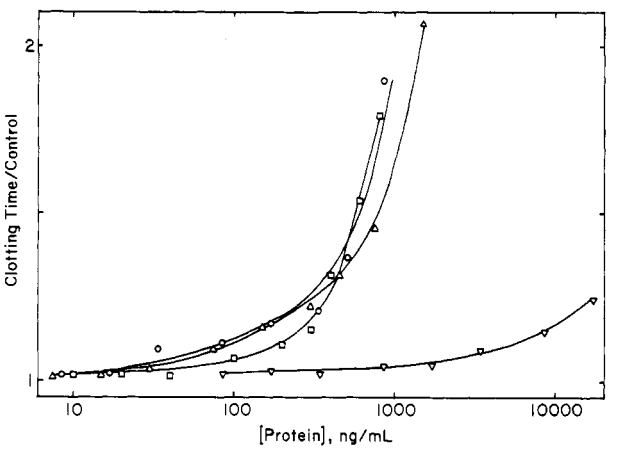

FIGURE 7: Inhibition of clotting by PAP-I, PAP-II, PAP-III, and PAP-IV. The activated partial thromboplastin time was determined in the presence of competitor protein as described under Experimental Procedures. Results are expressed as clotting times relative to clotting time in the absence of competitor protein. The final concentration of PC/PS (80/20) was 2.4 μM. Symbols are as in Figure 6.

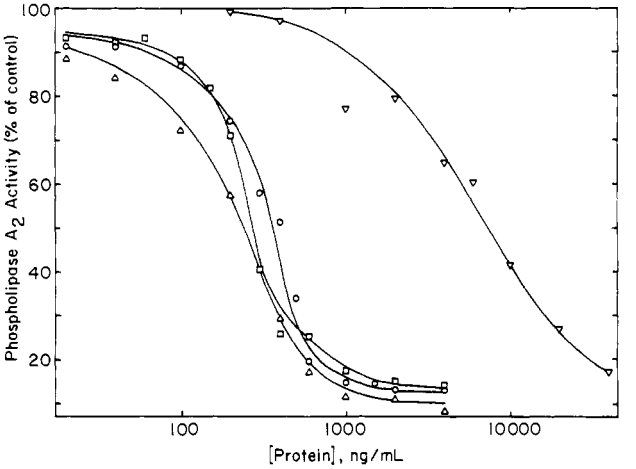

FIGURE 8: Inhibition of phospholipase A_2 activity by PAP-I, PAP-II, PAP-III, and PAP-IV. Phospholipase A_2 activity was measured in the presence of competitor protein as described under Experimental Procedures. The final concentration of C_6-NBD-PC/PS (80/20) was 3.1 μM. Enzyme activities are expressed as a percentage of the value obtained in the absence of competitor protein. Symbols are as in Figure 6.

fragment from PAP-IV was localized near the carboxyl terminus by sequence comparison. Thus, it is evident that PAP-IV is a cleaved product of lipocortin II.

Functional Characterization of Purified Proteins. We previously found that PAP-I binds with extremely high affinity to PC/PS vesicles in the presence of Ca^{2+} (Tait and Fujikawa, unpublished results). The binding of PAP-II, PAP-III, and PAP-IV to phospholipid vesicles was therefore studied under the same conditions previously used for PAP-I. These proteins, like PAP-I, all competed with FITC-PAP-I for binding to PC/PS vesicles (Figure 6); the binding was fully reversible upon addition of 5 mM EDTA (not shown). However, the potency of PAP-II (circles) and PAP-III (triangles) was somewhat less than that of PAP-I (squares), while PAP-IV (inverted triangles) was approximately 160-fold less potent. The potency of each protein was consistent from one preparation to another. Thus, while all four proteins bind tightly to phospholipid vesicles, their affinities and/or stoichiometries of binding are not identical.

The purified proteins were tested for anticoagulant potency in a standard partial thromboplastin time assay using synthetic phospholipid (PC/PS) (Figure 7). PAP-I, PAP-II, and PAP-III all had similar potencies, causing measurable prolongation of the clotting time at concentrations above 50 ng/mL; PAP-IV was much less potent, causing prolongation of the clotting time only at concentrations above 5000 ng/mL. The absolute and relative potencies of the four proteins closely paralleled their affinities for PC/PS vesicles (Figure 6).

The purified proteins were also tested as inhibitors of phospholipase A_2 (Figure 8). PAP-I inhibited phospholipase A_2, as previously shown (Haigler et al., 1987; Tait and Fujikawa, unpublished results); PAP-II and PAP-III had inhibitory potencies similar to PAP-I. PAP-IV/lipocortin II also inhibited phospholipase A_2 activity, as previously shown by others (Huang et al., 1986; Davidson et al., 1987). However, PAP-IV was again a far less potent inhibitor than PAP-I, PAP-II, or PAP-III, with an IC_{50} 30 times larger than PAP-I.

Discussion

We have described the isolation and characterization of three placental proteins (PAP-II, PAP-III, and PAP-IV) with anticoagulant activity in vitro. Both structural and functional studies establish that these proteins, like PAP-I, are members of the lipocortin family. Here we discuss the properties of these proteins, their relationship to similar or identical proteins described by other investigators, and the possible role of these proteins in blood clotting and other physiological processes.

Structural Aspects. PAP-III is a newly described member of the lipocortin family, while PAP-II is probably the human homologue of porcine protein II (Weber et al., 1987) or bovine endonexin (Geisow et al., 1986), and PAP-IV is a cleaved product of lipocortin II (Huang et al., 1986). Several criteria indicate that PAP-I, PAP-II, PAP-III, and PAP-IV are distinct proteins rather than precursors or degradation products of each other. Although their molecular weights are similar (Figure 2), their isoelectric points are quite distinct (Figure 3), they have different numbers of free sulfhydryl groups, and they do not cross-react with a polyclonal antibody to PAP-I (Figure 4). Antibody to PAP-II also does not cross-react with PAP-I, PAP-III, and PAP-IV (Miao et al., unpublished results). Finally, protein sequence data (Figure 5) show that PAP-II and PAP-III are structurally distinct from both PAP-I and lipocortins I and II.

The recent completion of sequences for PAP-I (Funakoshi et al., 1987b), protein II (Weber et al., 1987), and 67-kDa

Table I: Placental Proteins of the Lipocortin Family

	PAP-I[b]	PAP-II[b]	PAP-III[b]	PAP-IV[b]		
protein and synonyms[a]				lipocortin II calpactin I[d]	lipocortin I[c] calpactin II	
		protein II 32.5-kDa calelectrin		protein I 35-kDa calelectrin		protein III 67-kDa calelectrin
	endonexin II LRP-III[e]	endonexin LRP-V				LRP-IV
				p36 chromobindin 8	p35 chromobindin 6	p68 calcimedin
M_r (SDS–PAGE)	32000	33000	34000	34500	35000	67000
pI	4.8	6.1	5.9	8.1	7.9	
PC/PS binding (IC_{50}, ng/mL)	400	700	1300	68000		

[a] Some synonyms refer to apparently identical proteins from other tissues (see the introduction). Complete sequences are available for the underlined proteins. [b] Data for PAP-I, PAP-II, PAP-III, and PAP-IV are from this study. [c] Data for lipocortin I are from Huang et al. (1986) for the human protein. [d] Calpactin I refers to the heavy chain of the calpactin I tetramer. [e] LRP stands for lipocortin-related protein.

calelectrin or p68 (Sudhof et al., 1988; Crompton et al., 1988) allows a more extensive comparison of sequence similarities among members of the lipocortin family. These proteins all contain four internal repeats (Figure 5), except for 67-kDa calelectrin/p68, which contains eight. It is of particular interest for this study that PAP-I is more closely related to protein II/PAP-II (57% identity) than it is to lipocortins I and II (42% and 44% identity). This closer structural relationship between PAP-I and PAP-II may explain their greater functional similarity, as discussed below. The present protein sequence data (Figure 5) indicate that PAP-III has approximately the same percent identity with PAP-I, protein II/PAP-II, and lipocortins I and II. However, a definitive comparison will require a complete sequence.

Functional Aspects. PAP-II, PAP-III, and PAP-IV all bind tightly to PC/PS vesicles in the presence of Ca^{2+} (Figure 6), but they are not quantitatively identical with PAP-I. Under these assay conditions, protein concentrations causing 50% inhibition of binding (IC_{50}) were as follows (in nanograms per milliliter): PAP-I, 420; PAP-II, 730; PAP-III, 1340; PAP-IV, 68 000. Because of the extremely high affinity of the interaction between PAP-I and PC/PS vesicles, the observed IC_{50} values will be directly related to the phospholipid concentration used in the assay and will be far higher than the K_d. More extensive study will be necessary to investigate possible differences in the stoichiometry as well as the affinity of the binding reactions. It will also be necessary to investigate the reasons for the observed differences in the slopes of the inhibition curves for these four proteins (Figure 6); possible explanations include differences in cooperativity of binding or stoichiometry of binding. However, comparison of these proteins under identical conditions allows a relative ranking of apparent affinity by IC_{50} values, and from the present results, we can infer that PAP-II and PAP-III, like PAP-I (Tait and Fujikawa, unpublished results), bind to PC/PS vesicles with picomolar dissociation constants, while the K_d for PAP-IV/lipocortin II is much weaker.

PAP-I, PAP-II, PAP-III, and PAP-IV inhibit blood clotting (Figure 7) and phospholipase A_2 activity (Figure 8) with relative and absolute potencies very similar to their relative affinities for PC/PS. This supports the conclusion that the observed inhibitory effects are due in each case to phospholipid binding. All assays were performed with approximately the same concentration of phospholipid (2–3 μM) to allow direct comparison of results. Although PAP-I, PAP-II, and PAP-III have apparently equivalent potency in the clotting and phospholipase assays, these assays are not as precise as the phospholipid binding assay and hence do not reflect the relatively small differences in phospholipid binding affinity seen for PAP-I, PAP-II, and PAP-III. However, PAP-IV is much weaker that PAP-I, PAP-II, and PAP-III as an inhibitor of phospholipase A_2 and blood coagulation, which is consistent with its much lower affinity for phospholipid. These results are also consistent with an earlier study: lipocortins I and II both inhibited phospholipase A_2 with an IC_{50} of 4000 ng/mL when assayed with 0.5 μM phospholipid and 10 nM phospholipase A_2 (Davidson et al., 1987), as compared with an IC_{50} of 7600 ng/mL for PAP-IV/lipocortin II at 3.1 μM phospholipid and 21 nM phospholipase A_2 in the present study. It will be necessary to determine whether the differences observed with PAP-IV also occur with other phospholipid substrates in addition to PC/PS vesicles.

It seems most likely that inherent structural differences in the central 4-fold repeats of these proteins explain the lower potency of PAP-IV/lipocortin II relative to PAP-I, PAP-II, and PAP-III. PAP-IV is more distantly related to PAP-I than is PAP-II/protein II. As previously discussed (Funakoshi et al., 1987b; Weber et al., 1987), both PAP-I and protein II also have shorter amino-terminal extensions than lipocortins I and II. This amino-terminal extension is probably not important for in vitro inhibition of coagulation or phospholipase A_2, since PAP-I has the strongest phospholipid binding activity among the proteins of the lipocortin family. It has also been demonstrated that removal of a 3-kDa peptide from the amino terminus of lipocortin II had no marked effect on phospholipid binding (Glenney, 1986b; Johnsson et al., 1986b). This amino-terminal region, however, may be important in regulating other activities of these proteins in vivo, since it contains phosphorylation sites for protein–tyrosine kinases in lipocortin I (De et al., 1986; Pepinsky & Sinclair, 1986; Haigler et al., 1987) and lipocortin II (Glenney & Tack, 1985), and sites for protein kinase C in lipocortin II (Gould et al., 1986; Johnsson et al., 1986a) and protein II (Weber et al., 1987). In contrast to these proteins, PAP-I was not phosphorylatedd by either type of kinase in our preliminary studies (Funakoshi et al., 1987b).

Proteins of the Lipocortin Family. Previous sequence data have indicated the existence of at least five lipocortin-like proteins, and the present study adds a sixth member (PAP-III) to this family. These proteins have been purified from various tissues, but all of them are present in human placenta (Table I). Two methods have been used to purify these proteins from placenta: one uses the soluble fraction of an EDTA extract (Funakoshi et al., 1987a; Huang et al., 1986), while the other uses a particulate fraction prepared in the presence of Ca^{2+} (Haigler et al., 1987; Ahn et al., 1988). Previously, lipocortins I and II were isolated from the EDTA extract of placenta (Huang et al., 1986). In this and our previous study (Funa-

koshi et al., 1987a), we have purified four proteins (PAP-I, PAP-II, PAP-III, and PAP-IV/lipocortin II) from the EDTA extract; we did not find lipocortin I or intact lipocortin II in this fraction. At the same time as the present study, Ahn et al. (1988) isolated five lipocortin-related proteins from a particulate fraction of human placenta. Partial amino acid sequences showed that four of these proteins were identical with lipocortin I, lipocortin II, PAP-I, and PAP-II; lipocortin-related protein IV was the placental form of 67-kDa calelectrin (Sudhoff et al., 1988) or p68 (Crompton et al., 1988). During the purification of PAP-I, we also observed the presence of a high molecular weight anticoagulant activity in eluates from a gel filtration column (Funakoshi et al., 1987a). This protein is probably the same as lipocortin-related protein IV. More proteins of the lipocortin family may be present in placenta and could be isolated later. However, these proteins would probably be less abundant than the six proteins purified so far.

It should be mentioned that a protease, which is responsible for the cleavage of the amino-terminal region of these proteins, is present in placental cells. Reexamination of the amino-terminal sequence of PAP-I showed that ~5% of the protein was cleaved between Arg-5 and Gly-6, probably by a trypsin-like enzyme. It can be speculated that this enzyme also cleaves lipocortin II between Lys-10 and Leu-11; the newly formed amino-terminal Leu is then removed by an aminopeptidase to generate PAP-IV. Despite the inclusion of 0.5–5 mM benzamidine in the buffers used for homogenization of placental tissues and subsequent column chromatography, proteolytic degradation of these proteins was not prevented. More potent protease inhibitors will be required for isolation of the intact proteins.

Potential Physiological Significance. PAP-I is apparently the most abundant lipocortin-like protein purified from placenta, and it also binds most strongly to anionic phospholipid vesicles. The present study shows that at least three additional placental proteins from this family have anticoagulant activity in vitro. It is not yet known whether some or all of these proteins participate in physiological or pathological hemostasis in the placenta or elsewhere. It is possible that different members of this protein family may be specialized to serve different functions, such as exocytosis, membrane–cytoskeleton linkage, control of phospholipase activity, control of blood clotting, or perhaps other unsuspected functions. Further studies are under way to establish the structural, functional, and physiological properties of these proteins.

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REFERENCES

Ahn, N. N., Teller, D. C., Bienkowski, M. J., McMullen, B. A., Lipkin, E. N., & DeHaen, C. (1988) *J. Biol. Chem.* (in press).

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

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

Bridgen, P. J., Cross, G. A. M., & Bridgen, J. (1976) *Nature (London) 263*, 613–614.

Chen, P. S., Toribara, T. Y., & Warner, H. (1956) *Anal. Chem. 28*, 1756–1758.

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

Creutz, C. E. (1981) *Biochem. Biophys. Res. Commun. 103*, 1395–1400.

Creutz, C. E., Zaks, W. J., Hamman, H. C., Crane, S., Martin, W. H., Gould, K. L., Oddie, K. M., & Parsons, S. J. (1987) *J. Biol. Chem. 262*, 1860–1868.

Crompton, M. R., Owens, R. J., Totty, N. F., Moss, S. E., Waterfield, M. D., & Crumpton, M. J. (1988) *EMBO J. 7*, 21–27.

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

De, B. K., Misono, K. S., Lukas, T. J., Mroczkowski, B., & Cohen, S. (1986) *J. Biol. Chem. 261*, 13784–13792.

Edman, P., & Begg, G. (1967) *Eur. J. Biochem. 1*, 80–91.

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

Ericsson, L. H., Wade, R. D., Gagnon, J., McDonald, R. M., Granberg, R. R., & Walsh, K. A. (1977) in *Solid Phase Methods in Protein Sequence Analysis* (Previero, A., & Coletti-Previero, M. A., Eds.) pp 137–142, Elsevier, Amsterdam.

Fava, R. A., & Cohen, S. (1984) *J. Biol. Chem. 259*, 2636–2645.

Flower, R. J., & Blackwell, G. J. (1979) *Nature (London) 278*, 456–459.

Fujikawa, K., & McMullen, B. A. (1983) *J. Biol. Chem. 258*, 10924–10933.

Funakoshi, T., Heimark, R. L., Hendrickson, L. E., McMullen, B. A., & Fujikawa, K. (1987a) *Biochemistry 26*, 5572–5578.

Funakoshi, T., Hendrickson, L. E., McMullen, B. A., & Fujikawa, K. (1987b) *Biochemistry 26*, 8087–8092.

Gabriel, N. E., & Roberts, M. F. (1984) *Biochemistry 23*, 4011–4015.

Geisow, M. J. (1986) *FEBS Lett. 203*, 99–103.

Geisow, M. J., & Burgoyne, R. D. (1982) *J. Neurochem. 38*, 1735–1741.

Geisow, M. J., Fritsche, U., Hexham, J. M., Dash, B., & Johnson, T. (1986) *Nature (London) 320*, 636–638.

Gerke, V., & Weber, K. (1984) *EMBO J. 3*, 227–233.

Glenney, J. R., Jr. (1986a) *Proc. Natl. Acad. Sci. U.S.A. 83*, 4258–4262.

Glenney, J. R., Jr. (1986b) *J. Biol. Chem. 261*, 7247–7252.

Glenney, J. R., Jr., & Tack, B. F. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 7884–7888.

Glossmann, H., & Neville, D. M. (1971) *J. Biol. Chem. 246*, 6339–6346.

Gould, K. L., Woodgett, J. R., Isacke, C. M., & Hunter, T. (1986) *Mol. Cell. Biol. 6*, 2738–2744.

Haigler, H. T., Schlaepfer, D. D., & Burgess, W. H. (1987) *J. Biol. Chem. 262*, 6921–6930.

Hirata, F., Schiffmann, E., Venkatasubramanian, K., Salomon, D., & Axelrod, J. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 2533–2536.

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.

Iwasaki, A., Suda, M., Nakao, H., Nagoya, T., Saino, Y., Arai, K., Mizoguchi, T., Sato, F., Yoshizaki, H., Hirata, M., Miyata, T., Shidara, Y., Murata, M., & Maki, M. (1987) *J. Biochem. (Tokyo) 102*, 1261–1273.

Johnsson, N., Van, N. P., Soling, H.-D., & Weber, K. (1986a) *EMBO J. 5*, 3455–3460.

Johnsson, N., Vandekerckhove, J., Van Damme, J., & Weber, K. (1986b) *FEBS Lett. 198*, 361–364.

Kondo, S., Noguchi, M., Funakoshi, T., Fujikawa, K., & Kisiel, W. (1987) *Thromb. Res. 48*, 449–459.

Kristensen, T., Saris, C. J., Hunter, T., Hicks, L. J., Noonan, D. J., Glenney, J. R., & Tack, B. F. (1986) *Biochemistry 25*, 4497-4503.
Mann, K. G. (1984) *Prog. Hemostasis Thromb. 7*, 1-24.
Moore, P. B., & Dedman, J. R. (1982) *J. Biol. Chem. 257*, 9663-9667.
Nichols, J. W., & Pagano, R. E. (1981) *Biochemistry 20*, 2783-2789.
Pepinsky, R. B., & Sinclair, L. K. (1986) *Nature (London) 321*, 81-84.
Reutelingsperger, C. P. M., Hornstra, G., & Hemker, H. C. (1985) *Eur. J. Biochem. 151*, 625-629.
Saris, C. J. M., Tack, B. F., Kristensen, T., Glenney, J. R., Jr., & Hunter, T. (1986) *Cell (Cambridge, Mass.) 46*, 201-212.
Schlaepfer, D. D., Mehlman, T., Burgess, W. H., & Haigler, H. T. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 6078-6082.
Shadle, P. J., Gerke, V., & Weber, K. (1985) *J. Biol. Chem. 260*, 16354-16360.
Smith, V. L., & Dedman, J. R. (1986) *J. Biol. Chem. 261*, 15815-15818.
Sudhof, T. C., Walker, J. H., & Obrocki, J. (1982) *EMBO J. 1*, 1167-1170.
Sudhof, T. C., Ebbecke, M., Walker, J. H., Fritsche, U., & Boustead, C. (1984) *Biochemistry 23*, 1103-1109.
Sudhof, T. C., Slaughter, C. A., Leznicki, I., Barjon, P., & Reynolds, G. A. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 664-668.
Tait, J. F., Franklin, R. W., Simpson, J. B., & Ashwood, E. R. (1986) *Clin. Chem. (Winston-Salem, N.C.) 32*, 248-254.
Tamaki, M., Nakamura, E., Nishikubo, C., Sakata, T., Shin, M., & Teraoka, H. (1987) *Nucleic Acids Res. 15*, 7637.
Walker, J. H. (1982) *J. Neurochem. 39*, 815-823.
Walker, J. H., Obrocki, J., & Sudhof, T. C. (1983) *J. Neurochem. 41*, 139-145.
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., Plessmann, U., Van, P. N., Soling, H.-D., Ampe, C., & Vandekerckhove, J. (1987) *EMBO J. 6*, 1599-1604.
Wittenauer, L. A., Shirai, K., Jackson, R. L., & Johnson, J. D. (1984) *Biochem. Biophys. Res. Commun. 118*, 894-901.
Zwaal, R. F. A., Bevers, E. M., & Comfurius, P. (1986) in *Blood Coagulation* (Zwaal, R. F. A., & Hemker, H. C., Eds.) pp 141-169, Elsevier, Amsterdam.

Affinity Labels for the Anion-Binding Site in Ovotransferrin†

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ABSTRACT: Bromopyruvate, a known alkylating agent, has previously been reported to function as an affinity label for the anion-binding site in the iron-binding protein ovotransferrin [Patch, M. G., & Carrano, C. J. (1982) *Biochim. Biophys. Acta 700*, 217-220]. However, the present results indicate that hydroxypyruvate also functions in an almost identical manner, which implies that alkylation of a susceptible nucleophile cannot be the mechanism responsible for the covalent attachment of the anion. Model complexes and amino acid analysis of labeled ovotransferrin suggest that initial Schiff base formation, followed by reduction of the imine bond between the affinity anion and a lysine within the locus of the anion-binding site, accounts for the irreversible labeling. As expected, the covalently attached anions render the iron in the ovotransferrin–iron–anion ternary complex much more resistant to loss at low pH. It is proposed that the covalently labeled protein be used to test the hypothesis that iron removal from transferrin occurs by protonation and loss of the anion in low-pH lysosomal vesicles.

The term "transferrin" is applied to a whole class of iron-binding glycoproteins which includes the ovotransferrins, the lactoferrins, and the serum transferrins. The various transferrins are structurally and chemically quite similar, with the ability to tightly, but reversibly, bind 2 mol of ferric ion (Aasa et al., 1963). All have molecular masses of approximately 80 000 daltons and consist of single polypeptide chains of approximately 650 amino acids (Jeltsch & Chambon, 1982; MacGillivary et al.; Williams et al., 1982) folded into two compact regions, each of which can bind a single iron. An X-ray crystal structure of human lactoferrin has recently been published (Anderson et al., 1987). A number of excellent reviews on these important proteins are available (Aisen & Listowski, 1980; Chasteen, 1977, 1983a).

One of the unique features of the transferrins is that, in order for iron to bind, a suitable anion must also be present (Bates & Wernicke, 1971; Bates & Schlabach, 1973; VanSnick et al., 1973; Zweier et al., 1981). In physiological media, this necessary anion is carbonate (or possibly bicarbonate). In the absence of carbonate, numerous other organic anions can promote binding (Bates & Schlabach, 1973; Schlabach & Bates, 1975), although most simple inorganic anions are ineffective. The function of these synergistic anions may be to "lock in" the bound metal ion, thereby protecting it from

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