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Abstract: An aperture has been observed in the central canal at the filum terminale in some lower vertebrates and some mammals but not in the human. We examined 8 human spinal cords and 2 macaque monkey spinal cords and detected a caudal aperture in both human and monkey filum terminale. The ependymal lining of the human terminal ventricle was found to begin direct contact dorsally with the pia mater at 12.8 ± 5.3 mm caudal from the most cranial root of the 5th sacral nerve (S_5). In the human spinal cords the central canal was found to open into pia-arachnoid space at about 16.5 ± 5.0 mm caudal from S_5. Typical size of the caudal aperture was about 150 μm long × 130 μm wide. In the monkey filum terminale the caudal aperture appeared at about 45 mm caudal from S_5. The opening was about 100 μm long × 65 μm wide. The cytoplasmic process of the pial fibroblast was contiguous to the ependymal cell at the site of opening.

Key words: ventriculus terminalis, fine structure, cerebrospinal fluid, human, monkey

Introduction

Petromyzon and other species of lower vertebrates have long been known to have a caudal opening through which the central canal communicates with the subarachnoid space (Retzius, 1895; Kolmer, 1921). More recently, by light microscopy, the rhesus monkey and other mammals have been found to have an opening in the filum terminale (Wislocki et al. 1956), and by electron microscopy, the rabbit, rat, and guinea pig (Nakayama 1976).

In the human, after the terminal ventricle was defined by Krause (1875), many opinions were expressed concerning an opening from the central canal into subarachnoid space (Kramer, 1918; Streter, 1919). There was general agreement on the location of the terminal ventricle in the filum terminale and on its contact with the pia mater, but disagreement as to the opening. It was difficult to obtain a sample in good condition free from postomortem changes, and many researchers considered the opening an artefact.

For the present observation we acquired a sample, aong others, that provides a possible answer to the question. The sample was fixed within 10 minutes after cessation of circulation, and the tissue was adequately preserved for a light microscopic examination. As it was still not appropriate for electron microscopy, however, we chose the macaque monkey as representative of primates for observation of the caudal aperture under the electron microscope.

Material and Methods

Eight adult cadavers, male and female included, were fixed by infusion from the femoral artery with 20% formol and 37.5% phenol in 4,000 ml of 75% ethanol. Seven of these cadavers were donated for anatomical dissection, and one, case 7, was volunteered by a condemned criminal. Case 7 was fixed under legal authority within 10 minutes after cessation of circulation. Phenol was omitted from the fixative. The conus medullaris was cut at S_5, according to Kopsch (1930) and Crosby et al. (1962). From 7 cadavers, filum terminale together with the conus medullaris was dissected out and cut into 1 cm lengths. These were dehydrated and

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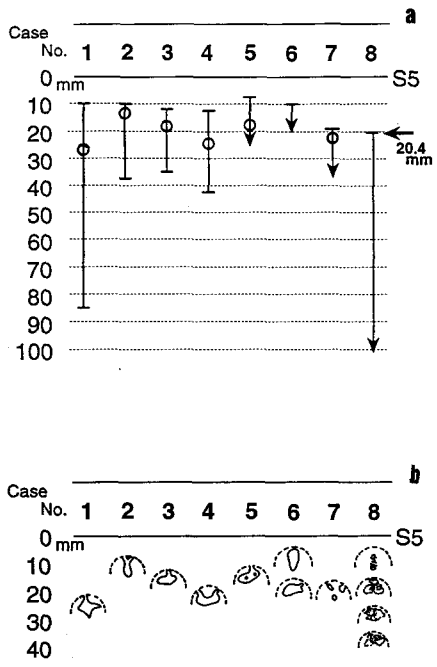
embedded in paraffin, keeping the original order and orientation. All pieces were cut into serial sections 6 μm thick, stuck on glass slides, deparaffined, hematoxylin-eosin stained and examined under a light microscope. Case 7 filum terminale with conus medullaris was dehydrated in toto and embedded in celloidin, then cut into serial section 25 μm thick, stained with hematoxylin and eosin, and examined under a light microscope. Images of central canal, terminal ventricle and pia mater were reconstructed using a computer (NEC 9801) operated with a three-dimensional analyze system (Olympus OZ).

Two Japanese monkeys (*Macaca fuscata*, 7.8 kg, male and 13.5 kg, male) were anesthetized by combined administrations of ketamine (20 mg/kg, subcutaneously) and pentobarbital (25 mg/kg, intraperitoneally). The animals were fixed by perfusion with 1,000 ml of 2% glutalaldehyde and 2% paraformaldehyde in 0.1 M cacodylate buffer (pH 7.4), and kept in 10% neutral formol. The spinal cord with filum terminale was removed from the vertebral column at the level of the 5th sacral nerve. The filum terminale together with the conus medullaris was further fixed in toto with 1% aqueous OsO_4 at 4°C. It was then cut into pieces 1 cm long, and these were dehydrated and embedded in Epon mixture, in original order and orientation. Thick sections were cut from the Epon blocks, stained with toluidine blue, and observed by a light microscope. Thin sections were stained with uranyl acetate and lead citrate, then examined with a Hitachi electron microscope at an accelerating voltage of 75 kV.



Diagrams 1a and b. (a) Levels and craniocaudal lengths of direct contact to the pia mater of the terminal ventricle (the pial covering) in 8 human spinal cords. Measurements are made from S_5. Circles indicate levels of the true openings. We did not examine sections more caudal from the level shown by the arrow. In case 8, the central canal was found to be almost obliterated at the S_4 level. The opening was not found in cases 6 and 9. (b) Schematical features of sections at levels of the opening. No true opening was observed in cases 6 and 8.

Fig. 1. Computer reconstructed images of two human spinal cords. A: Case 2. An image reconstructed by "smooth shading program" from 29 serial sections (6 μm thick) at 100 μm interval. Bracket shows approximate location of B. B: Case 7. An image reconstructed by "constant shading program" from 47 serial sections (25 μm thick) at 250 μm interval. Arrowheads indicate the levels of sections shown in Figs. 2 to 5. C, central canal; TV, terminal ventricle; P, pia mater.

Fig. 2. Light micrograph of a section indicated by the arrowhead 1 in Fig. 1B. Central canal (C) appears as a small white dot. PC, pial connective tissue. Bar, 200 μm.

Fig. 3. High power light micrograph of a section indicated by the arrowhead 2 in Fig. 1B. The ependymal cell lining ends at the sites shown by small arrows. A true orifice of about 130 μm (large arrow) is seen to communicate with the subarachnoid space (SS). Bar, 100 μm.

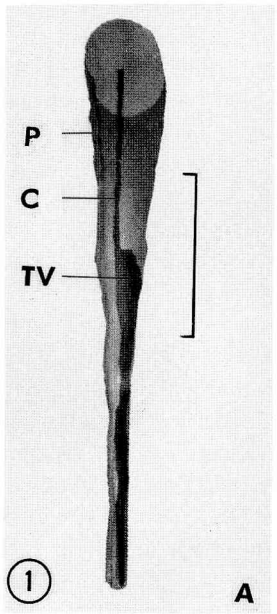
Fig. 4. Light micrograph, at the same magnification as Fig. 3, of a section indicated by the arrowhead 3 in Fig. 1B. The central canal (C) becomes Y-shaped, changing into the terminal ventricle, and the ventricular lumen opens (arrow) into the subarachnoid space (SS). P, pia mater; PC, pial connective tissue; Bar, 100 μm.

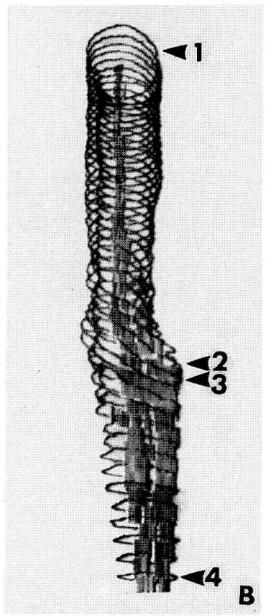
Fig. 5. Light micrograph of a section indicated by the arrowhead 4 in Fig. 1B. The Y-shaped central canal still remains (arrows). The main lumen of the central canal (C) is much larger than that in Fig. 2 to form the terminal ventricle. Bar, 200 μm.

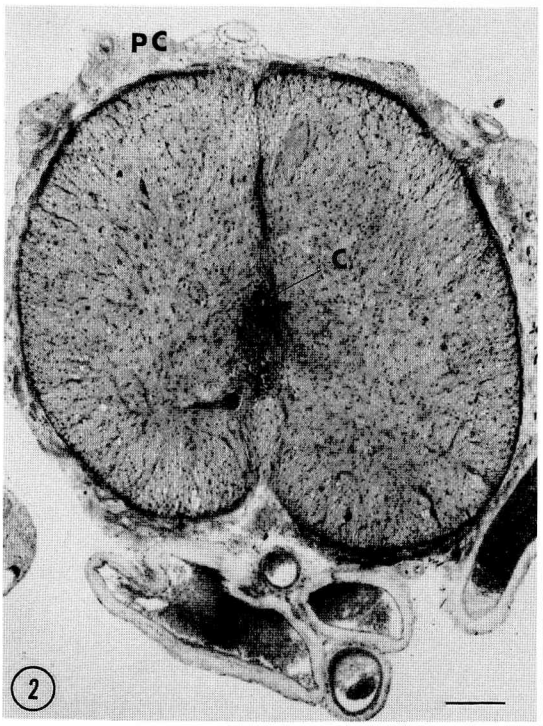
Results

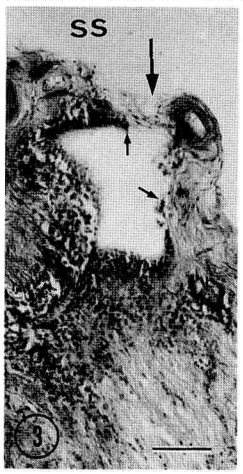
Human filum terminale

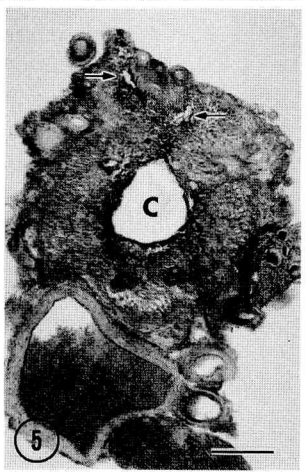
From the end of the conus medullaris, the central canal extends dorsally and forms the terminal ventricle. In our specimens the ventricular cavity shows remarkable variation in shape and size. The epndymal cell lining of the terminal ventricle was found to begin dorsal contact with











the pia mater at about 12.8 ± 5.3 mm caudal from S_5, and to end contact at distances varying from 35 mm to 100 mm or more from S_5. The true opening was found at about 16.5 ± 5.0 mm caudal from S_5 (Diagrams 1a and b). The opening, or caudal aperture, is about 150 μm long and 130 μm wide.

Computer reconstructed images of the conus medullaris, filum terminale, and central canal are shown in Fig. 1. In the conus medullaris and more cranial levels, the central canal is oval in cross section, and situated in the center of the spinal cord (Figs. 1 and 2). In more caudal levels, it is wider and Y-shaped as it comes to the terminal ventricle. The neural elements gradually diminish in amount, and the ependymal lining touches the overlying pia mater, opening eventually into the pia-arachnoid space (Figs. 3, 4 and 6). The terminal ventricle occupies the greater part of filum terminale (Fig. 5), then finally disappears as the ependymal cells separate from each other among connective tissue elements.

Monkey filum terminale

The true dorsal opening of the central canal into subarachnoid space, without the pial covering, was observed at about 45 mm caudal from S_5. The diameter of opening was about 65 μm (Fig. 7). The filum terminale was surrounded with fibroblasts and collagen fibers, but at the opening, ependymal cells, some with no cilia, turned out from the central canal and connected with fibroblasts in the pia mater. Figures 8a and b show a fibroblast on a common basal lamina with ependymal cells. Half desmosome-like densities were observed along the basal cell membrane of the fibroblast (Fig. 8b). Under light microscope, the central canal of the lower spinal cord often appeared compressed and obstructed (Fig. 9). Under the electron microscope, however, spaces were recognized among the microvilli and cilia (Figs. 10 and 11). Reissner's fiber and a dense material similar in fine structure to the Reissner's fiber (asterisk in Fig. 10) were observed in the central canal.

Discussion

Kernohan (1924) regarded the communication between the terminal ventricle and the subarachnoid space in human samples as an artefact. After him, it was generally accepted that in the human the central canal was closed and the cerebrospinal fluid hardly flowed. Bradbury et al. (1964) found that an Evans blue dye injected into the rabbit lateral ventricle stained not only the central canal but also the dorsal subarachnoid space around the filum terminale. Wislocki et al. (1956) with light microscope observed the aperture of the central canal opening into the meningeal space in the rhesus monkey. We confirmed this observation with electron microscope. In addition, our observations of the monkey spinal cord clearly showed that the central canal ending into the terminal ventricle was not occluded.

Nakayama (1976) was the first to have demonstrated with electron microscope the caudal opening of the central canal in rodents. The monkey causal aperture in our observations is very similar to those in the rat observed by Nakayama (1976). Sharing a common basal lamina, the cytoplasmic process of the pial fibroblast is contiguous to the ependymal cell at the opening. That is, mesodermal and ectodermal cells are contiguous to each other on a common basal lamina. The human caudal aperture, on the other hand, is rather similar to those in the guinea pig which opens towards the pia mater.

The Reissner's fiber and the dense material similar to it that we observed in the filum terminale support the speculation of Sterba et al. (1981) that these fibers, which are produced in the subcommissural organ (Gotow and Hashimoto, 1982; Nualart et al., 1991), pass unimpeded down the cnetral canal in the monkey spinal cord.

In this study, we fortunately observed one human spinal cord which was very suitable for light microscopy and clearly recognized a caudal opening in the central canal.

It is known that ependymal cells lack tight junctions. Macromolecular tracers such as horseradish peroxidase are known to pass through the zonulae adherentes and gap junctions of the ependymal layer (Brightman, 1969; Ogata et al., 1972). It is possible then, that the cerebrospinal fluid flowing caudally in the central canal may flow out not only through the site of direct contact with the pia mater, but more significantly through the caudal opening.

On the other hand, Oda and Nakanishi (1987)

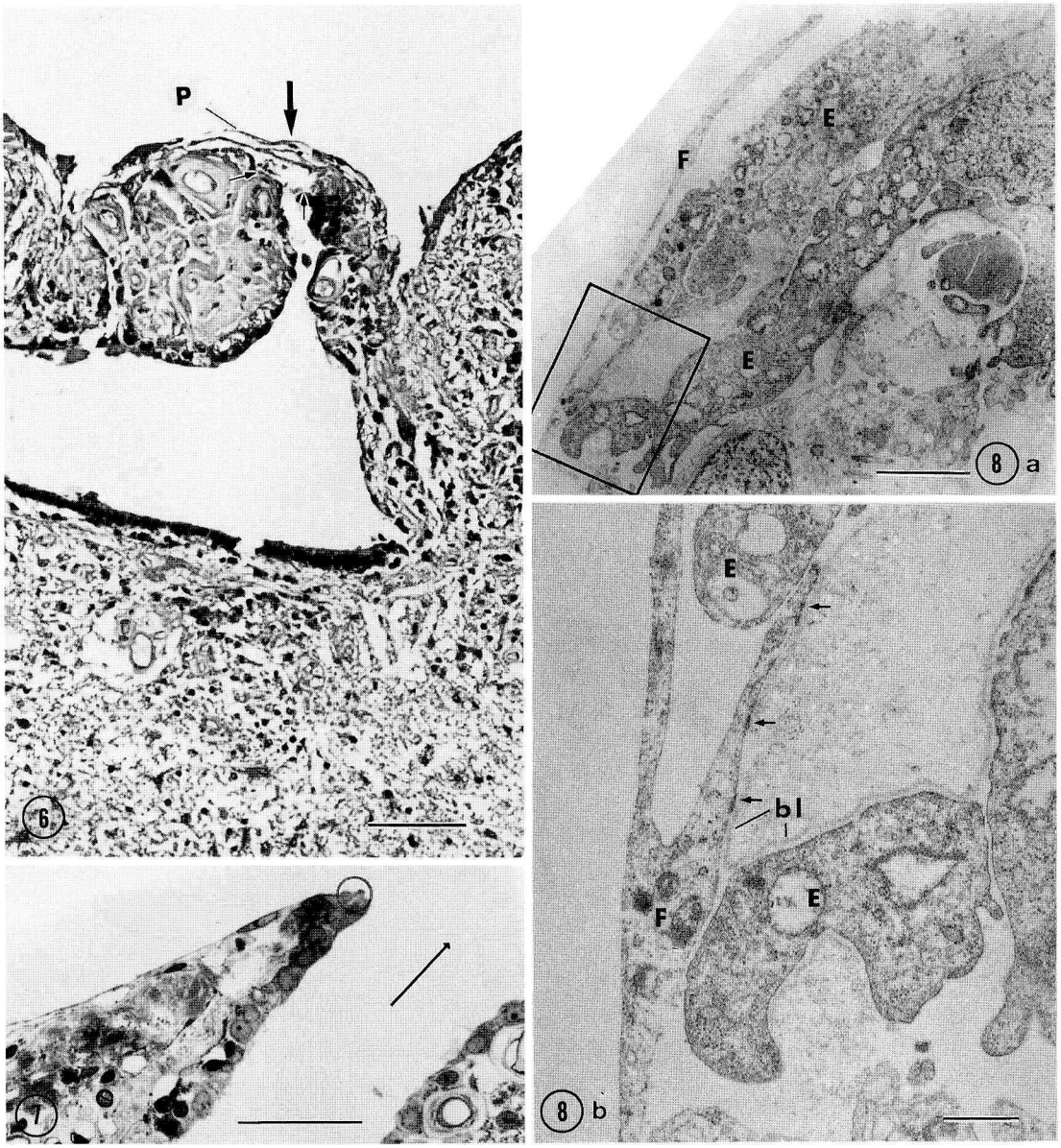


Fig. 6. Another sample (case 3) of the caudal aperture (large arrow) of human central canal, light micrograph. The ependymal cell lining ends at the sites shown by small arrow. P, pia mater; Bar, 100 μm.

Fig. 7. Light micrograph of a 1 μm cross-section (stained with toluidine blue) showing a dorsal opening of the monkey central canal. The opening is about 65 μm, in width. Arrow indicates dorsal direction. Circular area is enlarged in Fig. 8. Bar, 50 μm.

Fig. 8a. Electron micrograph of the circular area in Fig. 7. E, ependymal cells; F, pial fibroblast: Framed area is shown in Fig. 8b. Bar, 5 μm.

Fig. 8b. Higher magnification of the framed part in Fig. 8a. Tip of the fibroblast (F) ramifies and is contiguous with ependymal cells (E). These cells share a common basal lamina (bl). Half desmosome-like dense patches (arrows) are observed on the fibroblast plasma membrane facing the basal lamina. Bar, 1 μm.

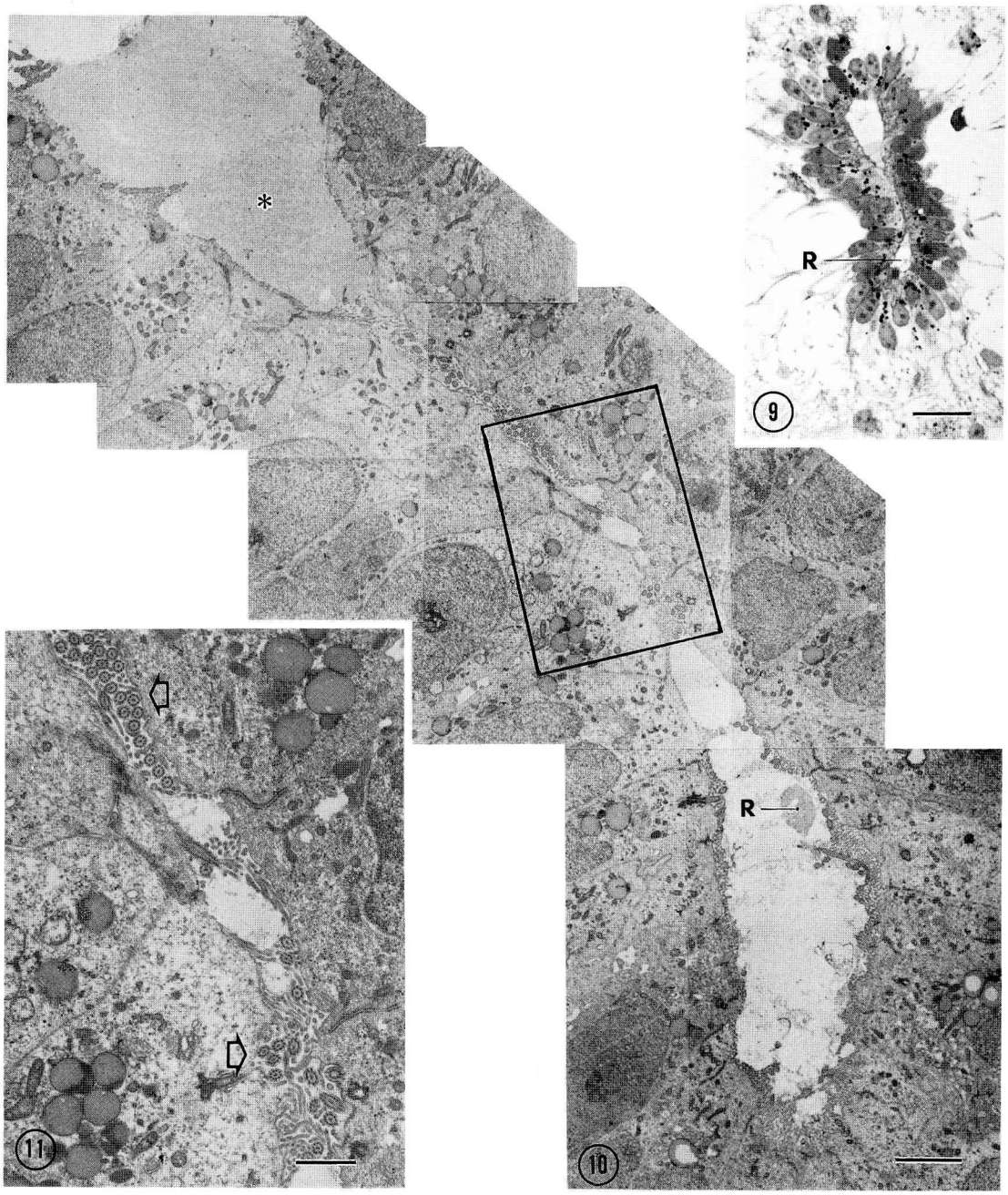


Fig. 9. Light micrograph of a 1 μm thick cross-section stained with toluidine blue showing a lower portion of the central canal in a monkey conus medullaris. The central canal looks partly compressed and obstructed. A tiny spot, seemingly a section of the Reissner's fiber (R) is seen. Bar, 50 μm.

Fig. 10. Electrom micrograph of a thin section adjacent to the section in Fig. 9. Dense material (*) fills the bottom of the dorsal half of the compressed central canal. This material is similar in fine structure to the Reissner's fiber (R). Framed area is enlarged in Fig. 11. Bar, 5 μm.

Fig. 11. Higher power observation of the framed part in Fig. 10. The compressed central canal is not obstructed but retains cerebrospinal fluid spaces among cilia and microvilli (open arrows). Bar, 2 μm.

suggested that in the mouse the membranous roof of the fourth ventricle would permit passage of the cerebrospinal fluid containing macromolecules via the ependymal cell clefts, implying that the foramen of Magendie would be not an anatomical aperture but a functional channel for the outflow of the cerebrospinal fluid. In any case the caudal aperture looks like those of Magendie and of Luschka of the fourth ventricle, and appears a secondary opening, not a direct descendant of the posterior neuropore.

References

1) Brightman MW, Reese TS (1969) Junctions between intimately apposed cell membranes in the vertebrate brain. J Cell Biol 40: 643–677
2) Bradbury MW, Davson H, Lathem W (1964) A flow of cerebrospinal fluid along the central canal of the spinal cord of the rabbit. J Physiol 172: 16P–17P
3) Crosby EC, Humphrey T, Lauer EW (1962) Correlative Anatomy of the Nervous System. Macmillan, New York
4) Gotow T, Hashimoto PH (1982) Intercellular junctions between specialized ependymal cells in the subcommissural organ of the rat. J Neurocytol 11: 363–379
5) Kernohan JW (1924) The ventriculus terminalis: Its growth and development. J Comp Nuerol 38: 107–125
6) Kolmer W (1921) Das "Sagittalorgan" der Wirbeltiere. Z Anat Entwicklungsgesch 60: 652–717
7) Kopsch FR (1930) Rauber-Kopsch Lehrbuch und Atlas der Anatomie des Menschen, Abt 5/6, Georg Thieme, Leipzig, pp 16–25
8) Krause W (1875) Der Ventriculus terminalis de Pückenmarks. Arch Mikrosk Anat 11: 216–230
9) Kramer SP (1918) The central canal of the spinal cord. Am J Insanity 75: 193–196
10) Nakayama Y (1976) The openings of the central canal in the filum terminale internum of some mammals. J Neurocytol 5: 531–544
11) Nualart F, Hein S, Rodriguez EM, Oksche A (1991) Identification and partial characterization of the secretory glycoproteins of the bovine subcommissural organ-Reissner's fiber complex. Evidence for the existence of two precursor forms. Brain Res, Mol Brain Res 11: 227–238
12) Oda Y, Nakanishi I (1987) Ultrastructure of the caudal portion of the fourth ventricular roof in the mouse. J Comp Neurol 256: 299–307
13) Ogata J, Hochwald GM, Cravioto H, Ranscohoff J (1972) Distribution of intraventricular horseradish peroxidase in normal and hydrocephalic cat brains. J Neuropathol Exp Neurol 31: 454–463
14) Retzius G (1895) Biologische Untersuchungen, Neue Folge 7, Das hinter Ende des Rückenmarkes und Chorda dorsalis bei Petromyzon fluviatillis. Gustav Fischer, Jena, pp 31–33
15) Sterba G, Kleim I, Nauman W, Petter H (1981) Immunocytochemical investigation of the subcommissural organ in rat. Cell Tissue Res 218: 659–662
16) Streter GL (1919) Factors involved in the formation of the filum terminale. Am J Anat 25: 1–12
17) Wislocki GB, Leduc EH, Mitchell AJ (1956) On the ending of Reissner's fiber in the filum terminale of the spinal cord. J Comp Neurol 104: 493–517

参考論文

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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 32000, 33000, 34000, and 34500, respectively. The presently estimated molecular weight of PAP-I is different from the previously determined value of 36500 (Funakoshi et al., 1987a). The reason for this discrepancy is not known. Nevertheless, the molecular weight of PAP-I was found to be 35847 from

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

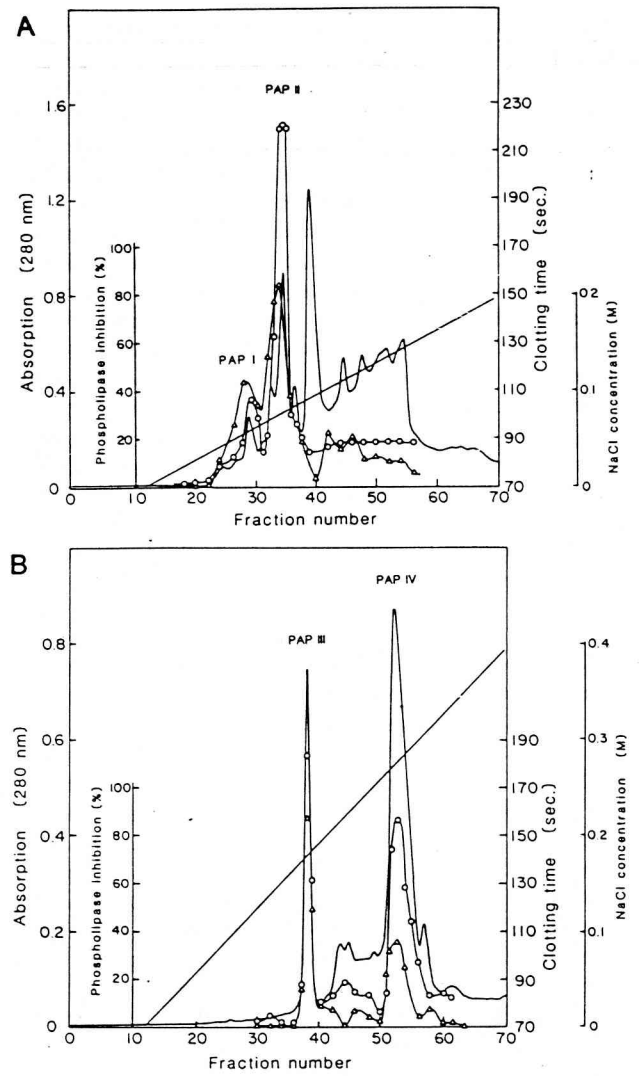

FIGURE 1: Final purification step of PAP-I, PAP-II, PAP-III, and PAP-IV by Mono S column chromatography. The pooled materials from the gel filtration column were prepared as described under Experimental Procedures and applied to a Mono S column connected to a Pharmacia FPLC system. The column was equilibrated 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

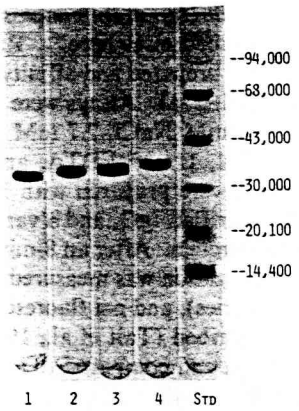

FIGURE 2: SDS–polyacrylamide gel electrophoresis of PAP-I, PAP-II, PAP-III, and PAP-IV. Reduced samples (5 μg) were applied to 7.5% acrylamide gels, and electrophoresis was carried out at 8 mA/tube for 3 h. Samples (from the left) were PAP-I (1), PAP-II (2), PAP-III (3), PAP-IV (4), and molecular weight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

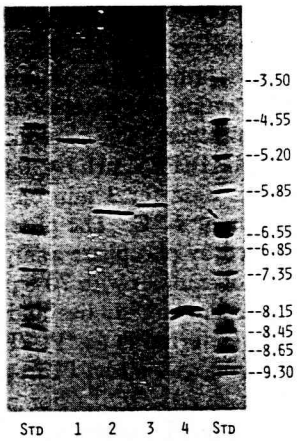

FIGURE 3: Isoelectric focusing of PAP-I, PAP-II, PAP-III, and PAP-IV. Approximately 0.5–1 μg of protein was applied to each lane; electrophoresis was then performed as described under Experimental Procedures. The isoelectric points of the standards are given at the right of the figure. Samples (from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

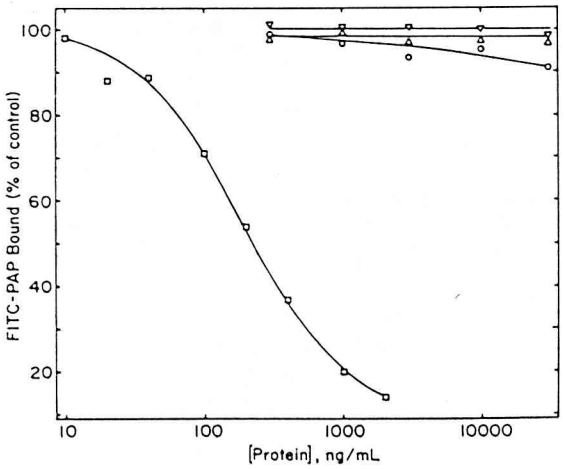

FIGURE 4: Immunological cross-reactivity of PAP-I, PAP-II, PAP-III, and PAP-IV with PAP-I antibody. Fluorescence polarization immunoassay was performed with varying amounts of unlabeled competitor protein as described under Experimental Procedures. Symbols: PAP-I (squares); PAP-II (circles); PAP-III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

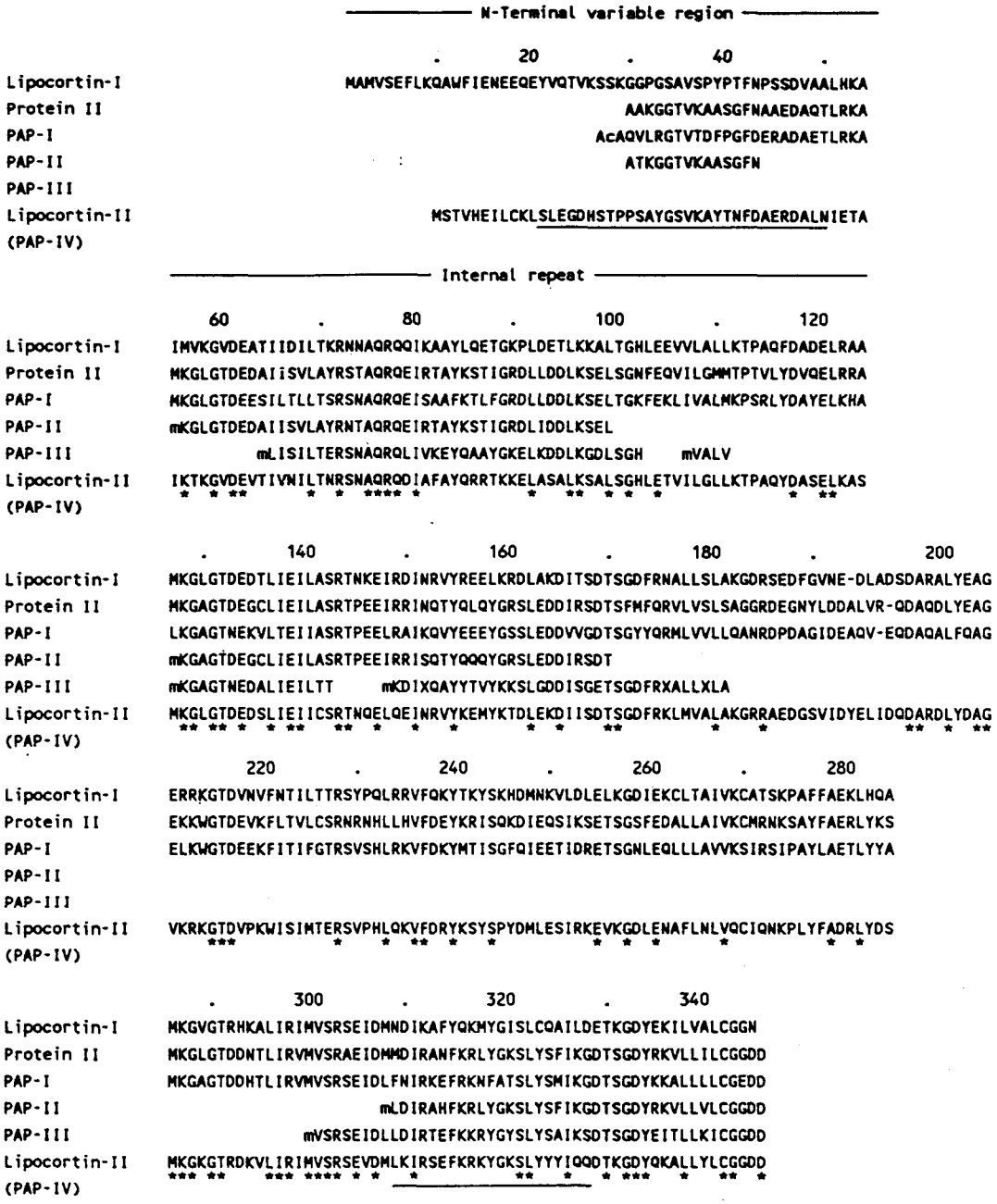

FIGURE 5: Amino acid sequences of cyanogen bromide fragments of PAP-II, PAP-III, and PAP-IV, and their alignments with PAP-I and lipocortins. Asterisks indicate conserved residues in the internal repeats. "m" represents expected methionine residues. "Ac" refers to an acetyl group. "X" indicates an unidentified 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

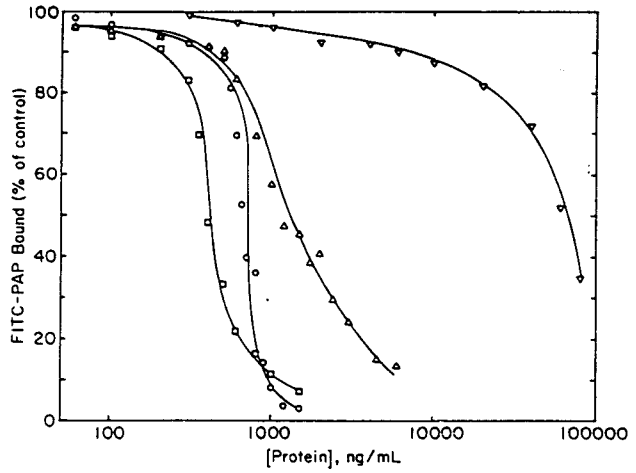

FIGURE 6: Binding of PAP-I, PAP-II, PAP-III, and PAP-IV to PC/PS vesicles. Binding assays were performed as described under Experimental Procedures by measuring the ability of each unlabeled protein to compete with FITC-PAP-I for binding to PC/PS vesicles. The final concentration of PC/PS (80/20) was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

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

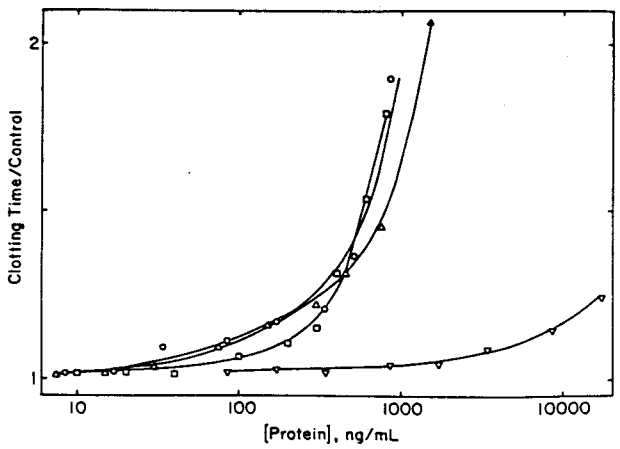

FIGURE 7: Inhibition of clotting by PAP-I, PAP-II, PAP-III, and PAP-IV. The activated partial thromboplastin time was determined in the presence of competitor protein as described under Experimental Procedures. Results are expressed as clotting times relative to clotting time in the absence of competitor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

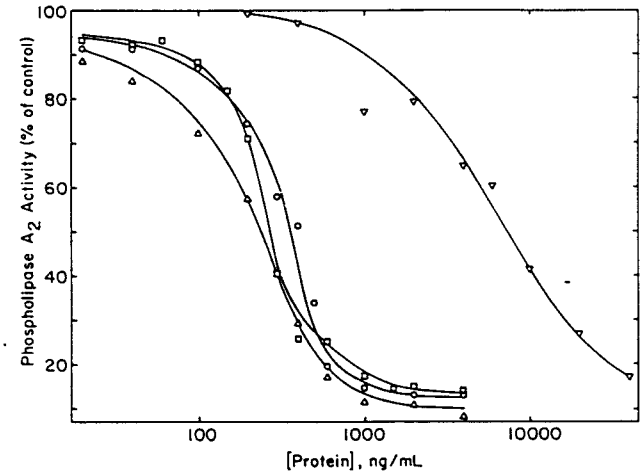

FIGURE 8: Inhibition of phospholipase A_2 activity by PAP-I, PAP-II, PAP-III, and PAP-IV. Phospholipase A_2 activity was measured in the presence of competitor protein as described under Experimental Procedures. The final concentration of C_6-NBD-PC/PS (80/20) was 3.1 μM. Enzyme activities are expressed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

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] protein I 35-kDa calelectrin	lipocortin I[c] calpactin II	
		protein II 32.5-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.

参考論文

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ヒト胎盤由来抗凝固蛋白質 (PAP-I) の局在性に関する研究

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臨床病理刊行会

ヒト胎盤由来抗凝固蛋白質（PAP-I）の局在性に関する研究

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Morphological Detection of Placental Anticoagulant Protein-I (PAP-I) in Human Placenta

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It has been reported that placental anticoagulant protein-I (PAP-I) inhibits the intrinsic and extrinsic pathways of blood coagulation and also the reconstituted prothrombinase activity. However, morphological study on the distribution of PAP-I in whole placenta or in placental cells has not been reported yet. We detected the PAP-I antigen, under light- and electron-microscope, by mean of immuno-cytological techniques. PAP-I was also present in microvilli of the placental syncytiotrophoblast cells and their cortical cytoplasm beneath the villi.

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[Key Words] placental anticoagulant protein（胎盤由来抗凝固蛋白質）, lipocortin family（リポコルチン族）, immunoelectron microscopy（免疫電顕）

血液凝固抑制物質としては，アンチトロンビンⅢのようにトロンビン・Xa 因子などのプロテアーゼ活性を抑制するもの[1]や，Va 因子・Ⅷa 因子を失活させる活性化プロテインCの生成に関与するトロンボモジュリン[2]など，数多く報告されている。われわれは最近，ヒト胎盤から抗凝固活性を持つ蛋白質を分離精製し報告した[3)~5)]。この蛋白質は，Iwasaki らによっても報告されている[6]。この胎盤由来抗凝固蛋白質（PAP-I）は，319 個のアミノ酸残基からなるリポコルチン族[7]に属する単純蛋白質であり，Ca^{2+} 存在下，酸性リン脂質に結合することにより，酸性リン脂質が関与するすべての凝固反応を阻害する。その抗凝固作用は，nanomolar の濃度で内因系[8]および外因系[8]凝固反応を抑制する強力なものである。また，ホスホリパーゼ A_2 の活性も阻害する。しかし，その生理的意義や細胞内での局在性は明らかでない。今回われわれは，PAP-I の胎盤における細胞内局在性を免疫組織化学的手法を用いて光学顕微鏡と電子顕微鏡下に観察した。

I. 方　法

A. PAP-I の精製

Funakoshi ら[3]の方法でヒト胎盤より精製純化した。この標品を還元し SDS ポリアクリルアミド電気泳動したものをクーマシイブリリアントブルーGで染色すると，分子量 36,500 の単一バンドを示した。

B. PAP-I 結合セファローズの作製

AFFI-GEL 15 (BIO-RAD 社) 5 ml に精製 PAP-

受理　1990年5月22日

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I (in pH 7.5 0.1 M 3-[N-Morpholino] propane-sulfonic acid) を加え，4°C で 15 時間ゆっくり攪拌し結合させた。PAP-I は，9.5 mg 結合した。

C. 抗 PAP-I 特異抗体の作製

0.3 mg の精製 PAP-I を Freund's complete adjuvant とともに3回家兎に免疫し，2カ月後に採血し抗 PAP-I 兎血清を得た。この抗 PAP-I 兎血清を PAP-I 結合セファローズカラムへ通し，抗 PAP-I 抗体をカラムに吸着させ，0.15 M NaCl を添加した 0.01 M リン酸緩衝液 pH 7.0 (P.B.S.) でカラムを充分洗浄した後，3 M NaSCN で溶出し，抗 PAP-I 特異抗体を得た。

D. 光学顕微鏡観察用ヒト胎盤標本の作製

出産直後のヒト胎盤を凍結切片とし，冷エタノールにて固定した。3％過酸化水素水を反応させ，内因性ペルオキシダーゼを破壊する。P.B.S. で洗浄，次に抗 PAP-I 特異抗体 (2.3 μg/ml in P.B.S.) に室温で 30 分間反応，洗浄後ペルオキシダーゼ結合抗兎 IgG 羊血清 (The Binding Site LTD. 社，England) 50 μg/ml (in P.B.S.) と 10 分間反応，洗浄し，ジアミノベンチジンと過酸化水素水で組織内局在 PAP-I と結合したペルオキシダーゼを発色検出した。対照染色は，メチルグリーンを用いて行った。

E. 電子顕微鏡観察用ヒト胎盤標本の作製

出産直後の胎盤を生理的食塩水で洗浄後，過ヨウ素酸・リジン・パラホルムアルデヒド (P.L.P.) 固定液にて 4°C 6時間固定，低温でアルコール脱水後，−20°C で4日間かけリジンに置換し，水溶性樹脂 (Lowicryl-K 4 M) に2日間 −20°C 下で紫外線重合にて包埋した。この試料を超薄切片としてニッケルグリッドに取り，P.B.S. で洗浄後，室温で抗 PAP-I 特異抗体 (2.3 μg/ml in P.B.S.) と3時間反応させた。洗浄液 (0.05% Tween 20 を含む P.B.S.) で充分に洗浄後，蛋白量にして 2.5 μg/ml (in P.B.S.)

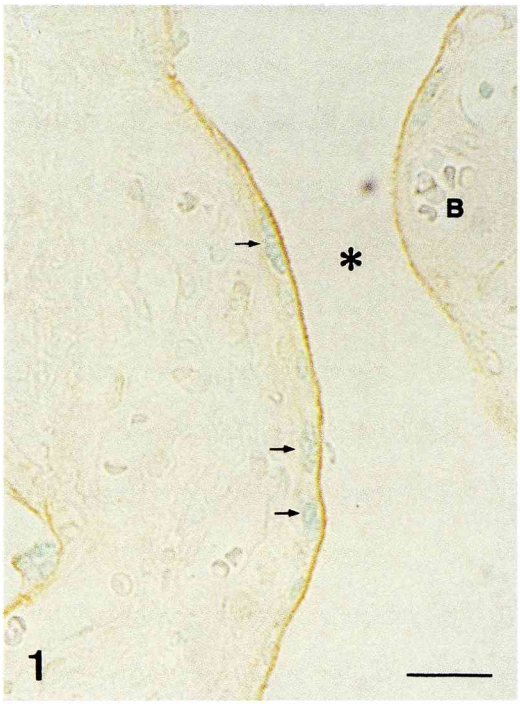

Figure 1 Light-microscopical photograph of two chorionic villi. Brown colored layer represents the existence of PAP-I.
Arrow : Nucleus of syncytiotrophoblast, * : Intervillus blood space, B : Fetal blood vessel, Bar : 20 μm.

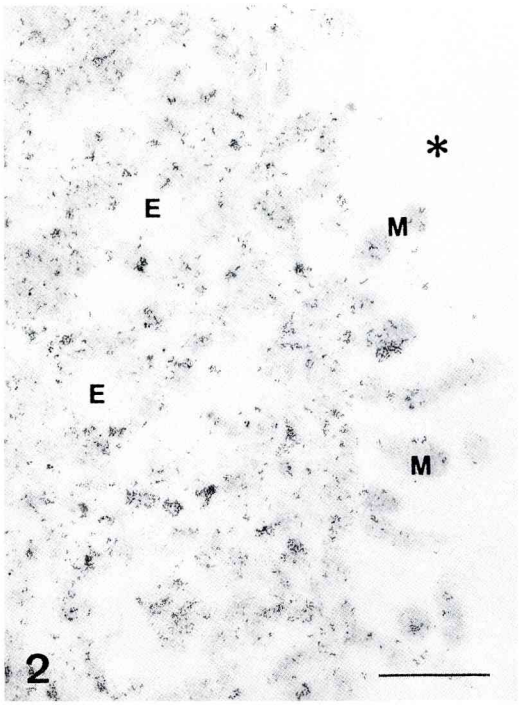

Figure 2 Electron-microscopical photograph with high magnification of the positive layer in **Fig. 1**. Colloidal-gold granules represent the existence of PAP-I.
* : Intervillous blood space, M : Microvilli, E : Endoplasmic reticulum, Bar : 0.5 μm.

の直径 5 nm 金コロイド粒子結合抗兎 IgG 山羊血清 (E・Y Lab 社, USA) と室温で5時間反応, 洗浄液で洗浄後, 2.5% 酢酸ウラン水溶液で単染色を行った。電子顕微鏡観察用ヒト胎盤標本の対照として, 抗 PAP-I 特異抗体のかわりに兎 IgG と反応させた後, 上記の金コロイド粒子結合抗 IgG を反応させた。

F. 電子顕微鏡観察

観察には, 日立 H 300 型電子顕微鏡を使用した。

II. 結果および考察

光学顕微鏡観察においては, **Fig. 1** に見られるごとく, 絨毛膜絨毛の絨毛間血液腔即ち母体血に面した栄養芽層合胞体細胞表層部にペルオキシダーゼ反応陽性部が観察できた。

電子顕微鏡を用いて, さらに細部を観察すると, **Fig. 2** に見られるごとく, 合胞体細胞の母体血と接する表層部および微絨毛への金コロイドの結合が観察された。対照の標本では, 金コロイドの吸着を認めなかった。

胎児の臓器である胎盤の絨毛間血液腔には母体血がゆるやかに流れており, その中に複雑に絨毛組織が入りこんでいるという胎盤の構造を考えると, 妊娠による凝固亢進状態[9] への胎盤の関与は大きいと考えられる。この胎盤の栄養芽層合胞体細胞表面に, 重要な凝固抑制物質のひとつであるトロンボモジュリンが存在する事が報告されている[10]が, このトロンボモジュリンは絨毛間血液腔において, トロンビンが産生されるとそれを捕捉し, プロテインCを活性化することで母体血が凝固するのを防いでいるのであろう。そして栄養芽層合胞体細胞内に存在する PAP-I は, 細胞が損傷すると遊出して, その機能を現わし, 他の凝固抑制物質とともに胎盤内での血液の凝固を抑制し流動性を保っていると考えられる。

III. ま と め

1) 胎盤由来抗凝固蛋白質 (PAP-I) の胎盤における細胞内局在を, 光学顕微鏡と電子顕微鏡を用い, 免疫組織化学的に検出した。

2) 栄養芽層合胞体細胞の母体血側表層部および微絨毛に陽性反応が検出された。

文 献

1) Rosenberg RD: Regulation of the hemostatic mechanism. The Molecular Basis of Blood Diseases, USA: W.B. Saunders, 1987. p 534～556

2) Esmon CT: The regulation of natural anticoagulant pathways. Science 235: 1348～1352, 1987

3) Funakoshi T, et al: Human placental anticoagulant protein; Isolation and characterization. Biochemistry 26: 5572～5578, 1987

4) Funakoshi T, et al: Primary structure of human placental anticoagulant protein. Biochemistry 26: 8087～8092, 1987

5) Tait JF, et al: Placental anticoagulant proteins: Isolation and comparative characterization of four members of the lipocortin family. Biochemistry 27: 6268～6276, 1988

6) Iwasaki A, et al: Structure and expression of cDNA for an inhibitor of blood coagulation isolated from human placenta: A new lipocortin-like protein. J Biochem 102: 1261～1273, 1987

7) Flower RJ: Lipocortins. Advances in Prostaglandin. Thromboxane and Leukotriene Research 17: 577～580, 1987

8) Kondo S, et al: Inhibition of human VIIa-tissue factor activity by placental anticoagulant protein. Thrombosis Research 48: 449～459, 1987

9) 真木正博：妊婦の出血傾向と凝固異常, 内科セミナー BLD 4 出血・凝固異常 (織田敏次, 他編). 大阪：永井書店, 1984. p 313

10) Maruyama I: Thrombomodulin is found on endothelium of arteries, veins, and lymphatics, and on syncytiotrophoblast of human placenta. The Journal of Cell Biology 101: 363～371, 1985

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