Studies on Novel Elastin-Binding Proteins in Human and Porcine Plasmas

1997. 3

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SUMMARY

Elastin is the core protein of elastic fibers and is a major component of the artery wall. Various constituents of blood, such as IgG, lipoproteins, calcium ions, and fibrin monomers, are known interact with elastin. The interactions denature elastin and decrease the elastic property of elastin. Their disregulated interactions may play significant roles in diseases of human aorta, such as arteriosclerosis and aneurysm.

In order to study the elastin-binding factors in blood, human plasma was applied to an α-elastin-Sepharose column. The column-binding fraction contained a 37-kDa protein, which was tentatively named EBP-37. Partial amino acid sequences of EBP-37 were determined. It had collagenous and non-collagenous domains. Homology searches of the sequences revealed that the protein is very similar but not identical to ficolin-α and -β, transforming growth factor-β1 (TGF-β1)-bindng proteins from porcine uterus membranes. The existence of oligomers and multimers crosslinked by disulfide bonds was demonstrated by immunoblot analysis. Direct interaction of EBP-37 with elastin was confirmed by demonstrating the binding of the isolated EBP-37 to α-elastin on a nitrocellulose membrane using the EBP-37-specific antiserum. In addition, it was found that a large amount of ficolin-like proteins is present in the α-elastin-Sepharose binding fraction of porcine plasma, and that recombinant porcine ficolin-α, as well as EBP-37, binds directly to elastin.

Recently, a novel human serum lectin with fibrinogen- and collagen-like domains, termed P35, was molecularly cloned and characterized. Comparison of the amino acid sequence of EBP-37 with that of P35 indicated that these proteins are identical. In addition, a novel corticosteroid-binding protein, termed hucolin, was purified from human plasma, and its amino-terminal amino acid sequence was found to be identical to that of EBP-37 and P35. Thus, EBP-37/P35/hucolin appears to function as a human ficolin.

Moreover, I obtained a cDNA that is closely related to, but different from, EBP-37/P35/hucolin by screening a human uterus cDNA library with porcine ficolin-α cDNA as a probe. This clone was termed human ficolin-1. Northern blot analysis of various human tissues revealed that human ficolin-1 mRNA is highly expressed in peripheral blood leukocytes. Taken together, these observations suggested that there are at least two ficolin-related proteins in both pig and human, which may have important roles as plasma proteins, and binding activity to elastin may be a common feature of the ficolin-related proteins.

Abbreviations: BSA, bovine serum albumin; CBB, Coomassie Brilliant Blue; ECM, extracellular matrix; FN, fibronectin; HLE, human leukocyte elastase; HRP, horseradish peroxidase; human S-GAL, spliced variant of human β-galactosidase; 36-kDa MAP, 36-kDa microfibril-associated glycoprotein; LTBP, latent transforming growth factor-β binding protein; MAGP, microfibril-associated glycoprotein; MBP, mannan-binding protein; MFAP4, a human microfibril-associated glycoprotein; MTN, human multiple tissue northern; PAE, porcine aortic endothelial; PBS, phosphate-buffered saline; PBST, 0.5% Tween-20 in PBS; PCR, polymerase chain reaction; pFN, plasma fibronectin; PPE, porcine pancreatic elastase; PVDF, polyvinylidene difluoride; SDS, sodium dodecyl sulfate; sheep EBP, sheep elastin-binding protein; TBS, Tris-buffered saline [0.1M NaCl-0.05M Tris-HCl (pH 7.4)]; TGF-β, transforming growth factor-β.

CONTENTS

INTRODUCTORY REMARKS 1

Chapter 1

Identification and Characterization of a Novel Elastin-Binding Protein (EBP-37) in Human Plasma

1-1 INTRODUCTION 11

1-2 MATERIALS and METHODS 12

1-3 RESULTS

1-3-1 Identification and Purification of a Novel Elastin-Binding Protein (EBP-37) in Human Plasma 16

1-3-2 Limited Proteolysis of EBP-37 and Amino Acid Sequence Analysis of Each Fragment and Amino-Terminal Amino Acid Sequence Analysis of EBP-37 18

1-3-3 Homology Searches of the Sequences and Comparisons of the Partial Sequences of EBP-37 with Ficolins 20

1-3-4 Existence of Oligomers and Multimers of EBP-37 21

1-4 DISCUSSION 24

Chapter 2

Identification and Characterization of EBP-37-Like Proteins (Porcine Plasma Ficolins) in Porcine Plasma

2-1 INTRODUCTION 26

2-2 MATERIALS and METHODS 26

2-3 RESULTS

2-3-1 Identification of EBP-37-Like Proteins (Ficolin-Like Proteins) in the α-Elastin-Binding Fraction of Porcine Plasma 28

2-3-2 Immunoblot Analysis of Porcine Plasma Ficolins and Distribution of Porcine Plasma Ficolin-Like Proteins in α-Elastin-Binding Fractions of Various Mammalian Plasmas 28
2-3-3 Amino Acid Sequencing of the 40-kDa and 37-kDa Proteins in α-Elastin-Binding Fraction of Porcine Plasma 30
2-4 DISCUSSION 33

Chapter 3
Binding of EBP-37 and Recombinant Ficolin-α to Elastin
3-1 INTRODUCTION 36
3-2 MATERIALS and METHODS 36
3-3 RESULTS
3-3-1 Direct Binding of EBP-37 to α-Elastin 38
3-3-2 Mode of Interaction of EBP-37 with α-Elastin 40
3-3-3 Elastin-Binding Activity of Recombinant Ficolin-α 40
3-4 DISCUSSION 43

Chapter 4
Molecular Cloning of Human Ficolin-1 and Expression of Human Ficolin-1 in Human Tissues
4-1 INTRODUCTION 47
4-2 MATERIALS and METHODS 47
4-3 RESULTS
4-3-1 Cloning of a Human Ficolin cDNA 49
4-3-2 Comparison of the Amino Acid Sequence of Human Ficolin-1 with Porcine Ficolins, EBP-37, Hucolin and P35 51
4-3-3 Expression of Human Ficolin-1 mRNA 55
4-4 DISCUSSION 55

Chapter 5

Interaction of Human Plasma Fibronectin and α-Elastin

5-1 INTRODUCTION 58

5-2 MATERIALS and METHODS 59

5-3 RESULTS

5-3-1 α-Elastin-Sepharose Affinity Chromatography of Human Plasma 62

5-3-2 SDS-PAGE and Immunoblot Analysis of α-Elastin-Sepharose Binding Proteins 62

5-3-3 Interaction of Purified pFN with α-Elastin-Sepharose 62

5-3-4 Binding of α-Elastin to pFN on Membranes 66

5-3-5 Alkyl-Sepharose Hydrophobic Chromatography of pFN 66

5-4 DISCUSSION 70

GENERAL DISCUSSION 72

ACKNOWLEDGEMENTS 77

REFERENCES 78

INTRODUCTORY REMARKS

A key step in the evolution of multicellularity must have been the ability of cells to contact tightly and interact specifically with other cells. Most of the cells in multicellular organisms are organized into cooperative assemblies called tissues, which in turn are associated in various combinations to form larger functional units called organs. The cells in tissues are usually in contact with a complex network of secreted extracellular macromolecules referred to as the extracellular matrix (ECM). The structural integrity of a tissue or organ is maintained by its ECM. Although matrix proteins participate actively in numerous aspects of cellular regulation, historically they have been defined by their physical and adhesive properties. This matrix helps to bind cells in tissues together and is a reservoir for many hormones controlling cell growth and differentiation. The matrix also provides an organized lattice through which cells can migrate and interact with one another, particularly during the early stages of differentiation. In many cases, the cells in a tissue are also held in place by direct cell-cell adhesions.

Cell-cell and cell-matrix junctions collectively are called cell junctions. Cell junctions occur at many points of cell-cell and cell-matrix contact in all tissues. Cell junctions can be classified into three functional groups: [1] occluding junctions, which can seal cells together in an epithelial cell sheet in a way that prevents even small molecules from leaking from one side of the sheet to the other; [2] anchoring junction, which mechanically attach cells (and their cytoskeletons) to their neighbors or to the extracellular matrix; and [3] communicating junctions, which mediate the passage of chemical or electrical signals from one interacting cell to its partner. The major kinds of cell junctions within each class are listed in Table 1 (1).

In the classification, I studied about the ECM, and especially concentrated on studies of elastin and elastin-binding proteins in human and porcine plasmas.

Table 1 A Functional Classification of Cell Junction (1)

1. Occluding junctions (tight junctions)

2. Anchoring junctions
   a. actin filament attachment sites
      i. cell-cell adherens junctions (e.g., adhesion belts)
      ii. cell-matrix adherens junctions (e.g., focal contact)
      iii. septate junctions (invertebrates only)
   b. intermediate filament attachment sites
      i. cell-cell (desmosomes)
      ii. cell-matrix (hemidesmosomes)

3. Communicating junctions
   a. gap junctions
   b. chemical synapses
   c. plasmodesmata (plants only)

Cytoskeleton, cell shape, cell migration, control of cell growth and differentiation, these are all subjects that, to be fully understood today, require a consideration of the ECM: its composition, role in development, and relationship to the cell surface. The macromolecules that constitute the ECM are mainly produced locally by cells in the matrix.

The ECM is the stable structural material that lies under epithelia and surrounds connective tissue cells, but the idea that the ECM is an inert supporting material, created by the cells as a mere scaffollding on or in which to reside, is now bygone. Certainly, collagens are a source of strength to the tissues, elastin is essential to matrix resiliency, and the structural glycoproteins help to create tissue cohesiveness. The cell, having produced these extracellular macromolecules and influenced their assembly in one way or another, dose not then divorce itself of them. The cell continues to interact with its own ECM products, and with the ECM produced by other cells (cell-matrix adherens junctions). At the cell surface, matrix receptors link the ECM to the cell interior; the

metabolism and fate of the cell, its shape, and many of its other properties are continuously related to and dependent on the composition and organization of the matrix.

The two main classes of extracellular macromolecules that make up the matrix are [1] polysaccharide chains of the class called glycosaminoglycans (GAGs), which are usually found covalently linked to protein in the form of proteoglycans, and [2] fibrous proteins of two functional types: mainly structural (for example, collagen and elastin) and mainly adhesive (for example, fibronectin and laminin). Glycosaminoglycan and proteoglycan molecules in connective tissue form a highly hydrated, gel-like "ground substance" in which the fibrous proteins are embedded; the polysaccharide gel resists compressive forces on the matrix, and the collagen fibers provide tensile strength. The aqueous phase of the polysaccharide gel permits the rapid diffusion of nutrients, metabolites, and hormones between the blood and the tissue cells; the collagen fibers both strengthen and help to organize the matrix, and rubberlike elastin fibers give it resilience. The adhesive proteins help cells attach to the appropriate part of the ECM: fibronectin, for example, promotes the attachment of fibroblasts and various other cells to the matrix in connective tissues, while laminin promotes the attachment of epithelial cells to the basal lamina.

Many vertebrate tissue, such as skin, blood vessels, and lungs, need to be both strong and elastic in order to function. A network of elastic fibers in the ECM of these tissues gives them the required resilience so that they can recoil after transient stretch. Long, inelastic collagen fibrils are interwoven with the elastic fibers to limit the extent of stretchig and prevent the tissue from tearing. Phylogenetic studies have shown that elastin occurs only in vertebrates and arose first in cartilaginous fish where its appearance coincides with the achievement of a fully closed circulatory system. The occurrence of elastin in both primitive and mordern sharks, and in chondrostean fishes suggests that the protein appeared in an early Devonian ancestor of all present-day fish, at some point after the divergence of the cyclostome and gnathostome lines (2).

In discussion of elastic tissues it is important to distinguish between "elastin" and "elastic fibers." The elastic fiber is the complex structure found in the ECM that contains elastin, microfibrillar proteins, lysyl oxidase, and, perhaps, proteoglycans. The main component of elastic fibers is elastin, a highly hydrophobic protein (about 750 amino acid residues), which, like collagen, is usually rich in proline and glycine but, ulike collagen, is not glycosylated and contains no hydroxylysine but small amounts of hydroxyproline are present.

Elastin molecules, named tropoelastin (the soluble secreted form of elastin), are secreted into the extracellular space and assemble into elastic fibers close to the plasma membrane, in cell-surface infoldings. After secretion the elastin molecules become highly cross-linked to one another to generate an extensive network of fibers and sheets. The cross-links are formed between lysine residues and important in imparting rubberlike properties to elastin.

A significant advance in the knowledge of elastin structure was the identification and characterization of cDNAs which encoded the elastin molecule. From the analysis of tryptic peptides derived primarily from porcine and chick tropoelastin, it was known that elastin contained segments enriched in hydrophobic amino acids as well as alanine-rich regions which incorporated several lysine residues that serve as cross-links. From these data, Gray *et al.* (3) predicted that the molecule was made up of alternating hydrophobic and cross-linking domains. The cDNA confirmed the prediction that the primary structure of tropoelasin consists of alternating hydrophobic and cross-linking domains (Fig.1) (4). The tropoelastin protein is composed largely of two types of short segments that alternate along the polypeptide chain- hydrophobic segments, which are responsible for the elastic properties of the molecule, and alanine- and lysine-rich α-helical segments, which form cross-links between adjacent molecules. Each segment is encoded by a separate exon. In the hydrophobic segment of bovine, porcine, and chick tropoelastins a

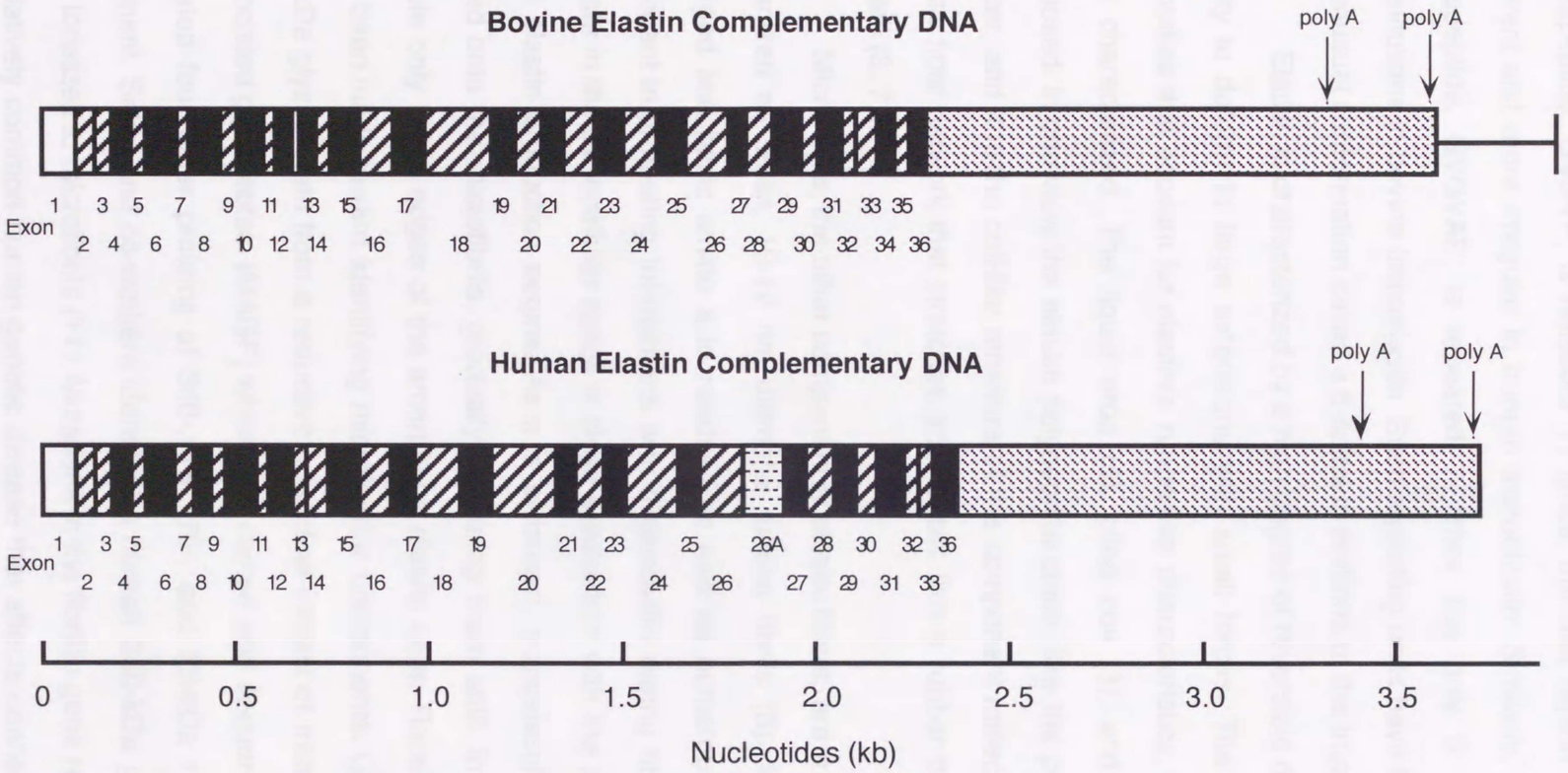


Fig. 1 Schematic diagram of cDNAs for bovine and human tropoelastin. The cDNAs are divided into exons which are numbered. Human exons are numbered relative to the bovine exons; note in the human gene the deletion of exons 33 and 34 and the addition of 26A which codes for a domain containing both hydrophobic and hydrophilic amino acids (4).

, hydrophobic sequences
, potential cross-linking sequences
, signal sequence
, 3'-untranslated region

pentapeptide, GVGVP, is repeated 11 times, but this repeat segment is considerably different and more irregular in human tropoelastin. Similarly, in human tropoelastin, a hexapeptide, GVGVAP, is repeated 7 times but only 5 times with conservative substitutions in bovine tropoelastin. Such repeating units have been postulated to confer an unusual conformation called a β-spiral in portions of the tropoelastin molecules.

Elastin is characterized by a high degree of reversible distensibility, including the ability to deform to large extensions with small forces. The precise physicochemical properties that account for elastin's rubberlike characteristics, however, have not been fully characterized. The liquid drop (5), oiled coil (3), and fibrillar models (6) are proposed. In one view the elastin polypeptide chain, like the polymer chains in ordinary rubber, and it is the coil-like structure of the component molecules cross-linked into the elastic fiber network that stretches and recoils like a rubber band (Fig.2: the oiled coil model) (3, 7).

Microfibrils, the other component of elastic fibers, are a complex of glycoproteins organized as small, 10-12 nm diameter tubular fibrils (8). Microfibrils contain many charged and basic amino acid residues as well as numerous cysteines that may be important in promoting interactions with tropoelastin during fiber assembly. Microfibrils appear in the extracellular space in close association with the plasma membrane before fetal elastin production begins. As it is produced, tropoelastin is secreted and cross-linked onto the microfibrils, gradually obscuring them until, in mature elastin, they are visible only at the edges of the amorphous elastin core. Recently, substantial progress has been made toward identifying microfibrillar components. Gibson *et al.* (9) isolated a 31-kDa glycoprotein from a reductive guanidine extract of microfibrils, called microfibril-associated glycoprotein (MAGP) which they cloned and sequenced (10). Moreover, they isolated four other proteins of 340-, 78-, 70-, and 25-kDa from fetal bovine nuchal ligament. Sakai and co-workers identified a human 350-kDa glycoprotein called fibillin that localizes to microfibrils (11). Mutations in the fibrillin gene result in Marfan syndrome, a relatively common human genetic disease that affects connective tissues that are rich

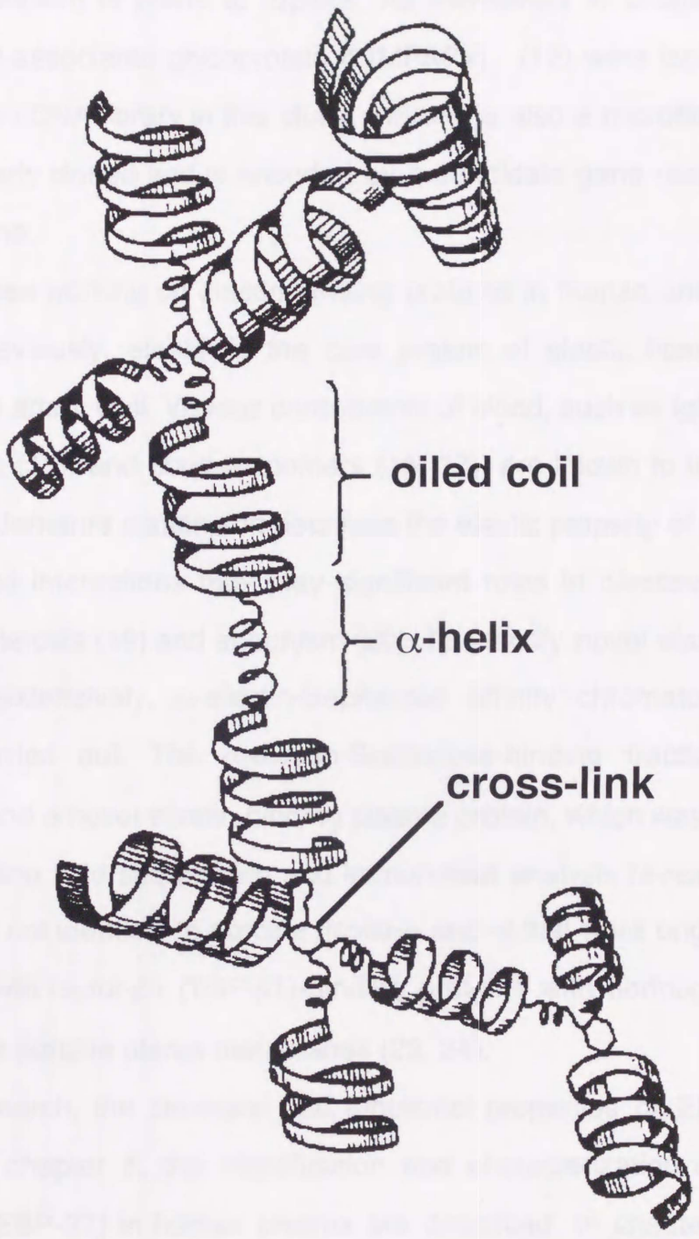


Fig.2 A proposed structure of elastin [3, 7]

in elastic fibers; in the most severely affected individuals, the aorta (whose wall is normally full of elastin) is prone to rupture. As mentioned in chapter 4, cDNAs for a human microfibril-associated glycoprotein 4 (MFAP4) (12) were isolated by screening the human uterus cDNA library in this study. MFAP4 is also a microfibril component. It is recently molecularly cloned and is encoded by a candidate gene responsible for Smith-Magenis syndrome.

I have been working on elastin-binding proteins in human and porcine plasmas. As described previously, elastin is the core protein of elastic fibers and is a major component of the artery wall. Various constituents of blood, such as IgG (13), lipoproteins (14), calcium ions (15), and fibrin monomers (16, 17), are known to interact with elastin. The interactions denature elastin and decrease the elastic property of elastin (Fig.3) (18). Their disregulated interactions may play significant roles in diseases of human aorta, such as arteriosclerosis (19) and aneurysm (20). To identify novel elastin-binding factors in blood more extensively, α-elastin-Sepharose affinity chromatography of human plasma was carried out. The α-elastin-Sepharose-binding fraction contained Igs, fibronectin (21) and a novel elastin-binding plasma protein, which was tentatively named EBP-37 (22). Amino acid sequencing and immunoblot analysis revealed that EBP-37 is highly similar but not identical to porcine ficolin-α and -β that were originally identified as transforming growth factor-β1 (TGF-β1)-binding proteins with fibrinogen- and collagen-like domains from porcine uterus membranes (23, 24).

In this research, the structural and functional properties of EBP-37 and ficolins were studied. In chapter 1, the identification and characterization of a novel elastin-binding protein (EBP-37) in human plasma are described. In chapter 2, presence and characterization of EBP-37-like proteins (porcine plasma ficolins) in porcine plasma are described. In chapter 3, it is confirmed that a main function of EBP-37 and porcine plasma ficolins is the elastin-binding activity. In chapter 4, it is described that human ficolin-1 cDNA encoding a human counterpart of porcine ficolins that is different from EBP-37 was cloned and expression of human ficolin-1 in human tissues. In addition, it is

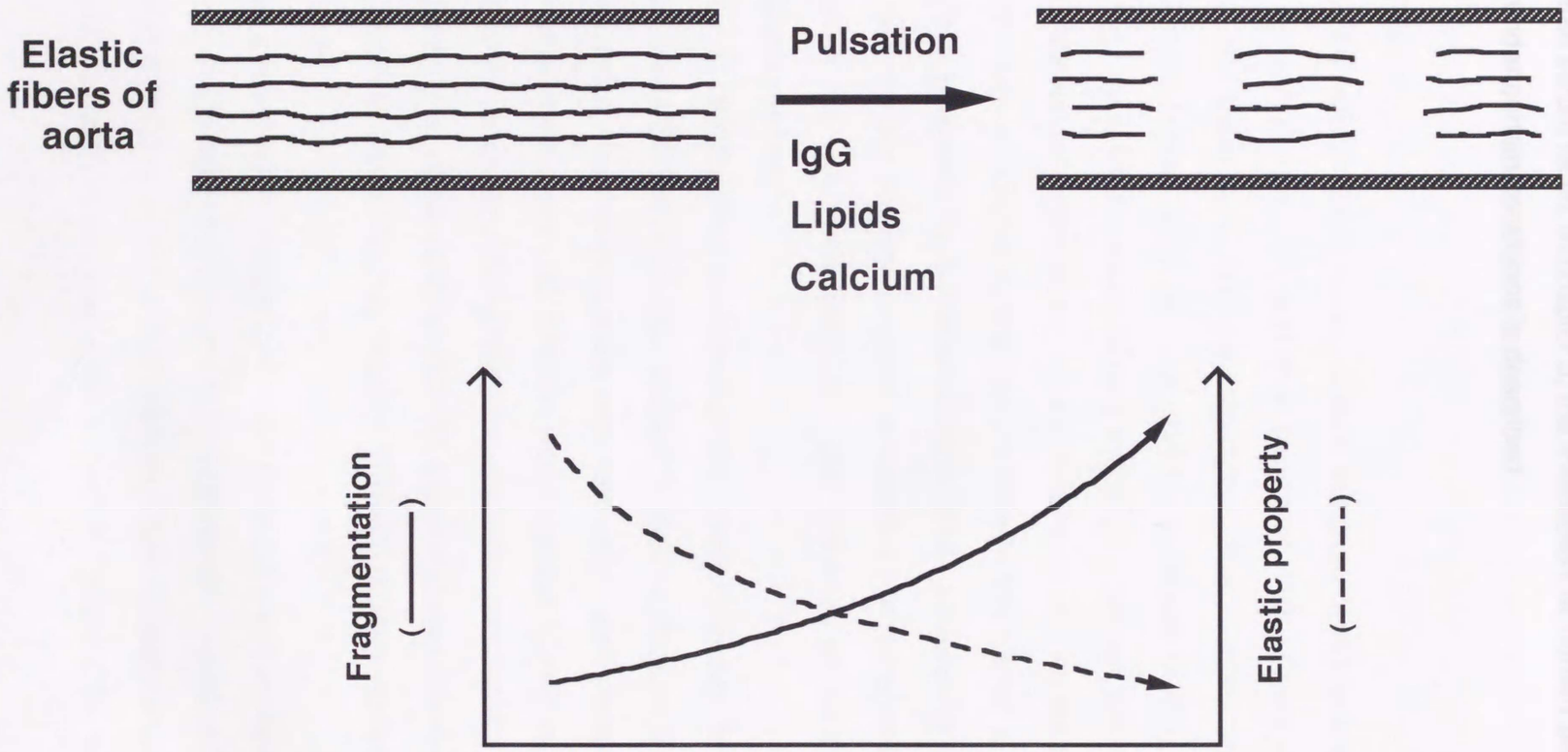


Fig.3 Interactions of plasma constituents with elastic fibers and fragmentation of elastic fibers (18).

mentioned that EBP-37 was identical to P35 that was recently cloned and characterized as a novel human serum lectin. In chapter 5, the interaction of human plasma fibronectin with α-elastin under certain conditions is described.

Chapter 1
Identification and Characterization of a Novel Elastin-Binding Protein (EBP-37) in Human Plasma

1-1 Introduction

As described in the introductory remarks, various constituents of blood such as Igs (13), lipoproteins (14), calcium ions (15), and fibrin monomers (16, 17) are known to interact with elastin and the interactions may play significant roles in arteriosclerosis (19) and aneurysm (20) of the human aorta. In order to study the elastin-binding factors in blood more extensively, we isolated elastin-binding proteins in human plasma by α-elastin-Sepharose 4B affinity chromatography. Since mature elastin is insoluble, I used α-elastin, an oxalic acid-solubilized form of elastin (25) in this study. α-Elastin contains lysyl-derived cross-links and structurally resembles mature insoluble elastin (26). The α-elastin-Sepharose binding fraction contained a number of proteins and the major ones were identified as Igs and fibronectin (21). The structures of other elastin-binding proteins remained unknown.

In chapter 1, I describe the identification and characterization of a novel elastin-binding protein named EBP-37 in human plasma. Sequence analysis indicated that it is very similar to ficolins, multimeric proteins with fibrinogen- and collagen-like domains from porcine uterus membranes (22). Ficolin was originally identified and purified as transforming growth factor β1 (TGF-β1)-binding proteins. Immunoblot analysis under nonreducing conditions indicated that EBP-37 forms dimers, trimers, tetramers and multimers by disulfide bonds. In this respect, EBP-37 is also similar to that of the recombinant ficolin.

Recently, a novel human serum lectin with fibrinogen- and collagen-like domains, termed P35 (27) was molecularly cloned and characterized. Comparison of the amino acid sequence of EBP-37 with that of P35 indicated that these proteins are identical. In addition, a novel corticosteroid-binding protein, termed hucolin (28), was purified from

human plasma, and its amino-terminal amino acid sequence was found to be identical to that of EBP-37 and P35.

Thus, EBP-37/P35/hucolin appears to function as a human ficolin, and may play important roles in view of its binding activities to diverse substances including elastin, sugar and corticosteroid.

1-2 MATERIALS and METHODS

MATERIALS

Sepharose 4B and molecular weight marker proteins (protein electrophoresis reagent HMW kit E and LMW kit E) were purchased from Pharmacia LKB Biotech. Human plasma was obtained from the Blood Center of the Japanese Red Cross. Chemicals for SDS-PAGE, CNBr, a silver stain kit and lysine peptidase *Achromobacter lyticus* protease I were from Wako Pure Chemical. Elastin from bovine neck ligament and 3,3'-diaminobenzidine tetrahydrochloride were from Sigma. Polyvinylidene difluoride (PVDF) membranes (Immobilon-P) were from Nihon Millipore Ltd.. Nitrocellulose membranes, a dot blotting system (Biodot) and a copper stain and destain kit were from Bio-Rad Laboratories. Glass fiber membranes (Glassybond membrane) were from Biometra. Centricon-10 and 30 were purchased from Amicon. *Staphlococcus aureus* V8 protease was purchased from Boehringer Mannheim. Horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG was purchased from Cappel research products. Plasma fibronectin was purified from normal human plasma by gelatin affinity chromatography (29). All other chemicals were of the purest grade commercially available.

α-Elastin-Sepharose 4B Affinity Chromatography of Human Plasma

Commercially obtained elastin was autoclaved several times or treated with hot alkali (30), and analyzed for amino acid composition. The results were consistent with

published data (31). α-Elastin, an oxalic acid-solubilized form of insoluble elastin,was prepared as described (25). Sepharose 4B gel was activated with CNBr. α-Elastin was then coupled to CNBr-activated-Sepharose 4B (21). Approximately 6mg of α-elastin was coupled per ml of hydrated gel. α-Elastin-Sepharose 4B affinity chromatography of human plasma was performed at 25°C (22). Human plasma containing 1mM phenylmethanesulfonyl fluoride was passed through a Sepharose 4B column and then applied to an α-elastin-Sepharose 4B column. After being washed, the column bound proteins were eluted with 5M urea-0.05M Tris-HCl (pH7.4 at 25°C). In the case of a stepwise manner elution, α-elastin-Sepharose bound proteins were eluted with 1M NaCl-0.05M Tris-HCl (pH7.4 at 25°C), 1M KBr-0.05M sodium acetate buffer (pH5.3 at 25°C) and 8M urea-0.05M Tris-HCl (pH7.4 at 25°C) as described previously (22).

Protein Determination

Protein concentrations were determined using a Bio-Rad protein assay kit (Bio-Rad Laboratories) or a BCA protein assay kit (Pierce Chemical Co.).

SDS-PAGE, Peptide Mapping and Electroblotting

The α-elastin-Sepharose binding fraction was subjected to SDS-PAGE (12.5% polyacrylamide gel) under reducing (1% 2-mercaptoethanol) or nonreducing conditions as described by Laemmli (32). Gels were stained with a silver stain kit .

Internal peptide sequences of the 37-kDa protein were obtained using the peptide mapping of Cleveland *et al.* (33) as follows. SDS-gels (the first and the second gels) were prepared at the preceding day (34) with the smallest quantity of ammonium persulfate. In the case of limited proteolysis with *Staphylococcus aureus* V8 protease , the second gel (15% polyacrylamide gel) contained 1mM EDTA. The α-elastin binding fraction was concentrated with a Centricon-30. The concentrated fraction was subjected to 12.5% polyacrylamide gel (the first gel) SDS-PAGE under reducing or nonreducing

conditions. After visualizing the 37-kDa bands by staining the gel with Coomassie Brilliant Blue (CBB) R-250 (Merck), the bands were excised.

When EBP-37 was digested with *Staphylococcus aureus* V8 protease, the bands were soaked for 10minx3 with vibration in a solution containing final concentration of 0.125M Tris-HCl, pH6.8, 0.1% SDS, 1mM EDTA and 1.5mM dithiothreitol. The sample wells of the second gel were filled with the same buffer and gel slices were pushed to the bottom of the well with a spatula. Spaces around the slices were filled by overlaying with 20μl of the buffer containing 20% glycerol. Finally, 10μl of the buffer containing 1.5μg of V8 protease, 10% glycerol and 0.005% bromophenol blue was overlaid into each slot and electrophoresis was performed in the normal manner with the exception that the current was turned off for 40min when the bromophenol blue dye neared the bottom of the stacking gel.

When the EBP-37 was digested with lysyl endopeptidase (*Achromobacter lyticus* protease I), peptide mapping was performed in the same manner with the exception that EDTA and dithiothreitol were eliminated from buffers and 10μl of enzyme solution contained 1μg of lysyl endopeptidase.

Electroblotting of peptide fragments of EBP-37 from SDS-gel to a Glassybond membrane (35) was performed as described by Towbin *et al.* (36) and the manufacture's instruction. Electrophoretic transfer was performed with a Multiphor II Electrophoresis System (Pharmacia Biotech). The membrane was stained with CBB R-250. The peptide bands that were judged to be suitable for sequencing were excised.

In the case of amino-terminal amino acid sequencing of the 37-kDa protein, electroblotting of the α-elastin-binding proteins of human plasma from SDS-gel (12.5% polyacrylamide gel, nonreducing conditions) onto a Glassybond membrane was performed as described. The membrane was stained with CBB R-250. The peptide bands that were judged to be suitable for sequencing were excised.

Sequence Analysis

The amino acid sequences were determined with a pulse-liquid phase amino acid sequencer (477A protein sequencer, Applied Biosystems).

Sequence Homology Search

Sequence homology was searched in the PIR, SWISS-PROT protein data base, GenBank and EMBL data base using Intelligenetics Suite (IntelliGenetics).

Isolation of EBP-37

In order to raise the polyclonal antibody to EBP-37 and examine the interaction of EBP-37 with α-elastin, EBP-37 was extracted from the bands of SDS-gels. The concentrated α-elastin-Sepharose binding fraction was boiled for 4min or treated at 4°C overnight with the SDS-PAGE sample buffer. It was subjected to SDS-PAGE (12.5% polyacrylamide gel) under nonreducing conditions. After visualizing the 37-kDa bands by staining the gel with CBB R-250 or copper stain and destain kit, the bands were excised. EBP-37 was extracted from the bands in the SDS electrophoresis buffer at 4°C. The extract was dialyzed against PBS, 100mM potassium phosphate buffer and PBS, successively. The extracted EBP-37 was used as the antigen and for the experiment of the interaction with α-elastin.

Preparation of the EBP-37 Antiserum

About 100μg (0.5ml) of the extracted EBP-37 mixed with an equal volume of Freund's complete adjuvant (Wako Pure Chemical) was injected into a female rabbit (New Zealand White, 2kg) followed by the injection of a further 100μg of the extracted EBP-37 three times with at 10 days intervals (boosters). The antiserum was prepared.

Immunoblot Analysis of EBP-37

The a-elastin-Sepharose-binding fraction of human plasma was mixed with SDS sample buffer, boiled at 95°C for 4 min or treated at 4°C overnight, and subjected to SDS-PAGE

(7.5% polyacrylamide gel) under nonreducing or reducing conditions. After electrophoresis, the proteins were transferred to PVDF membranes. The membrane was washed with phosphate-buffered saline (PBS) containing 0.5% Tween-20 (PBST) for 5min three times, and then soaked in PBS containing 3%(w/v) BSA (pH7.3) overnight at 4°C. It was washed with PBST three times, and reacted with the EBP-37 antiserum or preimmune serum, diluted 1/4000 in 3% BSA in PBS. HRP (horseradish peroxidase)-conjugated goat anti-rabbit IgG diluted 1/2000 in the same solution was used as a second antibody. 0.05% 3,3'-Diaminobenzidine, 0.045% H_2O_2 and 0.84mM $CoCl_2$ in 10mM Tris-HCl, pH7.5, were used to detect the second antibody.

1-3 RESULTS

1-3-1 *Identification and Purification of a Novel Elastin-Binding Protein (EBP-37) in Human Plasma*

Human plasma was passed through a Sepharose 4B column and then applied to an α-elastin-Sepharose 4B column at 25°C. The binding fraction was subjected to SDS-PAGE. Fig.4 shows the patterns of SDS-PAGE (12.5% polyacrylamide gel) under reducing (Fig.4 A) and nonreducing (Fig.4 B) conditions. A clear band of a 37-kDa protein was found besides the bands derived from Igs (22). It was not found in the eluates from the plain Sepharose 4B (data not shown). As described below, sequence studies indicated that the 37-kDa band under reducing conditions and that under nonreducing conditions are derived from the same protein. This protein was named EBP-37. Compared with the migration of EBP-37 under reducing conditions, it migrated slightly faster and gave a sharp band under nonreducing conditions. EBP-37 was extracted from the excised 37-kDa SDS-PAGE bands under nonreducing conditions. It gave a single band with SDS-PAGE (Fig.4 C).

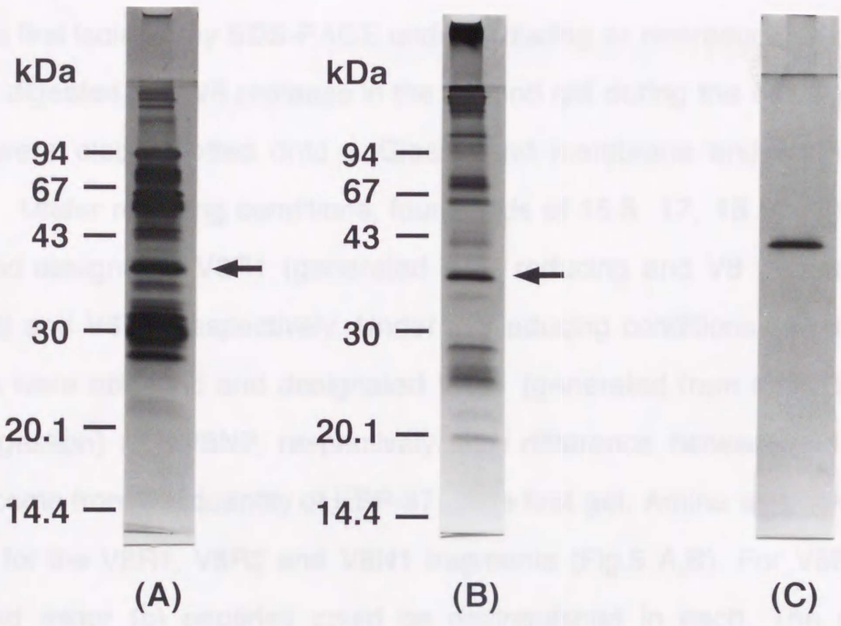


Fig.4. SDS-PAGE and silver staining of α-elastin-Sepharose-binding proteins and isolated EBP-37.
Aliquots (15μl) of α-elastin-Sepharose-binding fraction, which had an absorbance at 280nm value of 0.35 were subjected to SDS-PAGE under reducing (A) and nonreducing (B) conditions. The 37 kDa band was excised and extracted as described in the text and subjected to SDS-PAGE under nonreducing conditions (C). The positions of the molecular weight markers are shown on the left. The arrows indicate the 37-kDa protein.

1-3-2 *Limited Proteolysis of EBP-37 and Amino Acid Sequence Analysis of Each Fragment and Amino-Terminal Amino Acid Sequence Analysis of EBP-37*

EBP-37 was first isolated by SDS-PAGE under reducing or nonreducing conditions. The protein was digested with V8 protease in the second gel during the electrophoresis. The fragments were electroblotted onto a Glassybond membrane and stained with CBB (Fig.5 A,B). Under reducing conditions, four bands of 15.5, 17, 18 and 20.5 kDa were obtained and designated V8R1 (generated from reducing and V8 protease digestion), V8R2, V8R3 and V8R4, respectively. Under nonreducing conditions, two bands of 15.5 and 17 kDa were obtained and designated V8N1 (generated from nonreducing and V8 protease digestion) and V8N2, respectively. The difference between the two patterns seemed to come from the quantity of EBP-37 in the first gel. Amino acid sequences were determined for the V8R1, V8R2 and V8N1 fragments (Fig.5 A,B). For V8R1 and V8R2, major(a) and minor (b) peptides could be distinguished in each. The sequences of V8R1(a), V8R2(a) and V8N1 were identical and a collagenous sequence. The 13th and 16th amino acid residues in V8R1(a) were shown to be hydroxyproline residues. The sequences of V8R1(b) and V8R2(b) were non-collagenous sequences.

Then EBP-37 was isolated under nonreducing conditions and was digested with lysyl endopeptidase in the second gel during the electrophoresis. The fragments were electroblotted onto a Glassybond membrane and were stained with CBB (Fig.5 C). Three bands of 16, 17 and 23 kDa were obtained and designated LN1 (generated from nonreducing and lysyl endopeptidase digestion), LN2 and LN3. Amino acid sequences of the LN1(a), LN2 and LN3 were identical and a collagenous sequence, but differed from that of V8R1(a). The fourth amino acid residue in these peptides was shown to be a hydroxyproline residue. LN2 peptide contained a short non-collagenous sequence besides the collagenous sequence. The sequence of LN1(b) was a non-collagenous sequence, and identical to that of V8R1(b).

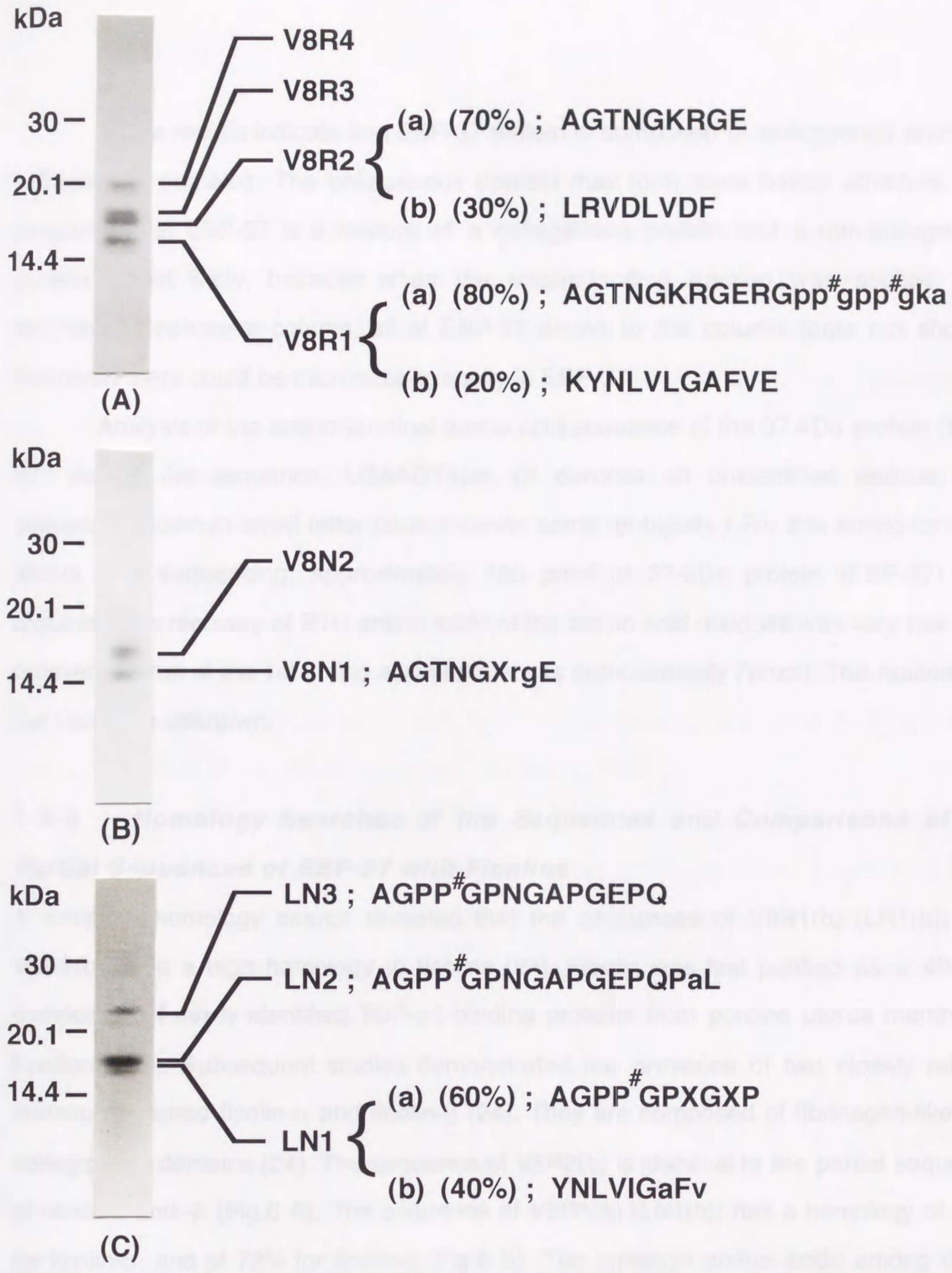


Fig.5. Amino acid sequence analysis of each proteolytic fragment.
Lanes (A), V8 protease digests of the reduced protein; lane (B), V8 protease digests of the nonreduced protein; lane (C), lysyl endopeptidase digests of the nonreduced protein. Details of digestion procedures are given in the text. When a band contained a mixture of peptides, the percentage of the total accounted for as each peptide (a,b) is indicated (as determined at cycle 1). P#(p#) denotes a hydroxyproline residue. The two characteristic peaks of the residue in sequence analysis were found to coincide with those appeared on the analysis of a hydroxyproline residue contained in an authentic chemically synthesized peptide. X denotes an unidentified residue. The sequence of small letter code possesses some ambiguity. The positions of the molecular weight markers are shown on the left.

These results indicate that EBP-37 protein is composed of collagenous and non-collagenous domains. The collagenous domain may form triple helical structure. The possibility that EBP-37 is a mixture of a collagenous protein and a non-collagenous protein is not likely, because when the elastin-binding fraction was applied to a fibronectin-Sepharose column, all of EBP-37 bound to the column (data not shown). However, there could be microheterogeneity in EBP-37.

Analysis of the amino-terminal amino acid sequence of the 37-kDa protein (EBP-37) yielded the sequence, LQAADTXpe. (X denotes an unidentified residue. The sequence shown in small letter code involves some ambiguity.) For this amino-terminal amino acid sequencing, approximately 100 pmol of 37-kDa protein (EBP-37) was required. The recovery of PTH amino acids of the amino acid residues was very low (The recovery of that of the 1st amino acid residue was approximately 7pmol). The reasons for the result are unknown.

1-3-3 *Homology Searches of the Sequences and Comparisons of the Partial Sequences of EBP-37 with Ficolins*

A computer homology search revealed that the sequences of V8R1(b) (LN1(b)) and V8R2(b) have a high homology to ficolins (24). Ficolin was first purified as a 40-kDa component of newly identified TGF-β1-binding proteins from porcine uterus membrane fractions (23). Subsequent studies demonstrated the presence of two closely related molecules named ficolin-α and ficolin-β (24). They are composed of fibrinogen-like and collagen-like domains (24). The sequence of V8R2(b) is identical to the partial sequence of ficolin-α and -β (Fig.6 A). The sequence of V8R1(b) (LN1(b)) has a homology of 90% for ficolin-α, and of 72% for ficolin-β (Fig.6 B). The common amino acids among these sequences are well conserved. These sequences were not related to those of known plasma proteins that have collagenous domains such as C1q (37) mannan-binding protein (MBP) (38, 39), conglutinin (40) and collectin-43 (41).

As for collagenous sequences, the sequence of V8R1(a) (V8R2(a), V8N1) have a homology of 72% for ficolin-β, and also 81% for procollagen α1(I) chain-human (fragment) (42) (Fig.6 C). The sequence of LN2 (LN1(a) and LN3) have a homology of 66% for ficolin-α, and of 73% for cuticle collagen 13 precursor (43) (Fig.6 D). The sequence of LN2 seems to be the junction of collagenous and non-collagenous regions. The homology of sequences of collagenous region between EBP-37 and ficolins is not high as compared with that of non-collagenous region.

The amino-terminal six-amino acid sequence of EBP-37 coincided with that of a 36-kDa protein that was identified as one of three soluble class I human leukocyte antigen proteins in human plasma by immunoprecipitation and immunoblotting using anti-human leukocyte antigen monoclonal antibodies (44). Of the three proteins, the amino-terminal amino acid sequences of two species were identical to that of cellular human leukocyte antigen, whereas no homology was found for the other 36-kDa protein. It was possible that the 36-kDa protein is identical to EBP-37.

When EBP-37 was identified in human plasma (22), these results indicated that EBP-37 is a new plasma protein with elastin-binding activity and very similar to ficolins which are found in porcine uterus membranes and presence of ficolins in plasma has not been reported. As described below, two human ficolin gene products have been identified afterward. The products were identical to EBP-37.

1-3-4 *Existence of Oigomers and Multimers of EBP-37*

α-Elastin-Sepharose binding fraction was subjected to SDS-PAGE under nonreducing conditions, and then immunoblot analysis was carried out (Fig.7). High molecular weight materials and four molecules with sizes of 150, 108, 75 and 37 kDa reacted with the EBP-37 antiserum. They were converted to 37 kDa molecule upon reduction. These results indicate the existence of disulfide-linked multimers, tetramers, trimers and dimers

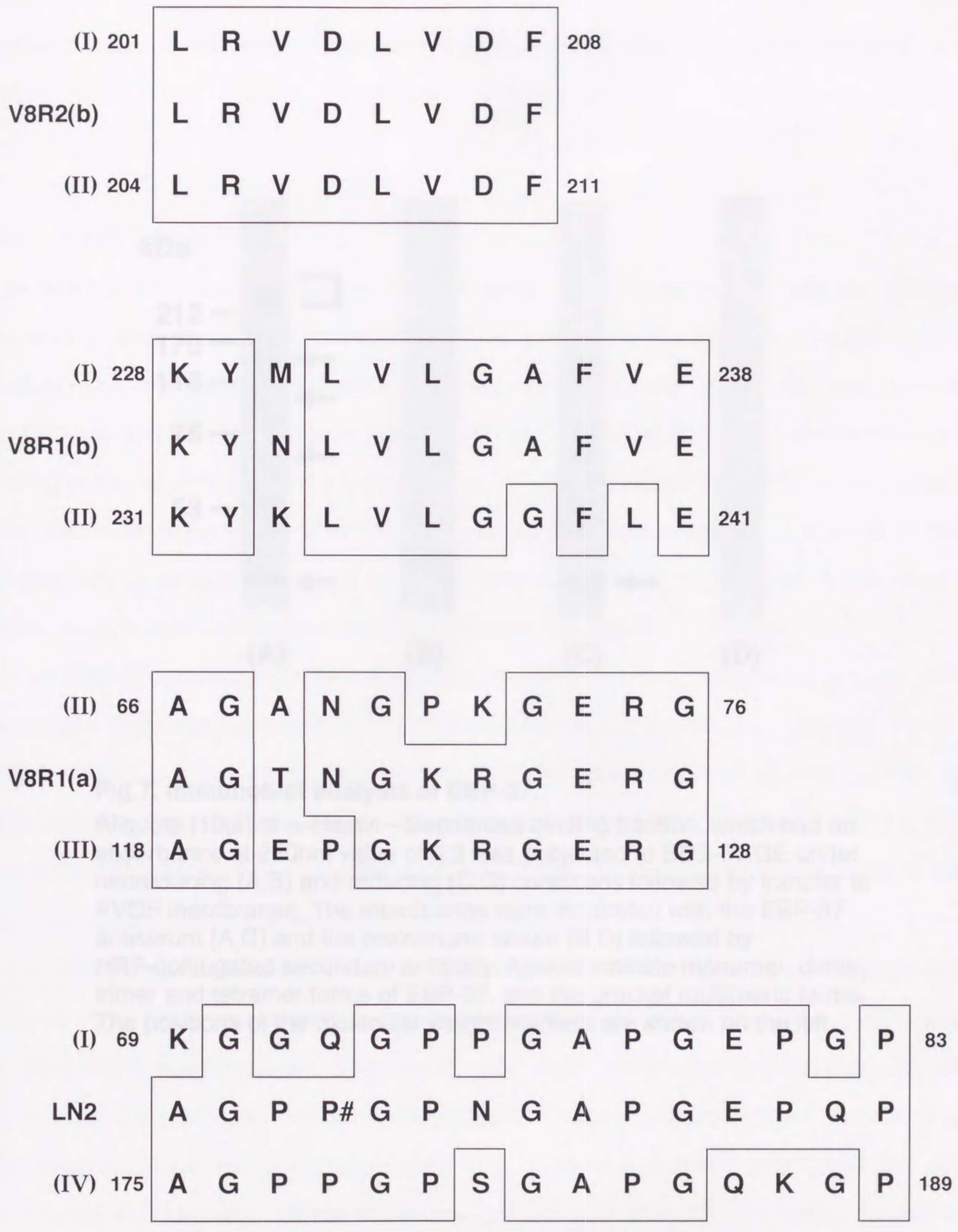


Fig.6. Amino acid sequence homology between EBP-37 and other known proteins. Comparisons of the partial amino acid sequences of ficolin-α (I), -β (II), procollagen α1(I) chain-human (fragment) (III), cuticle collagen 13 precursor (IV) and proteolytic fragments from EBP-37. P# denotes a hydroxyproline residue. Identical amino acid residues are boxed. Details are given in the text.

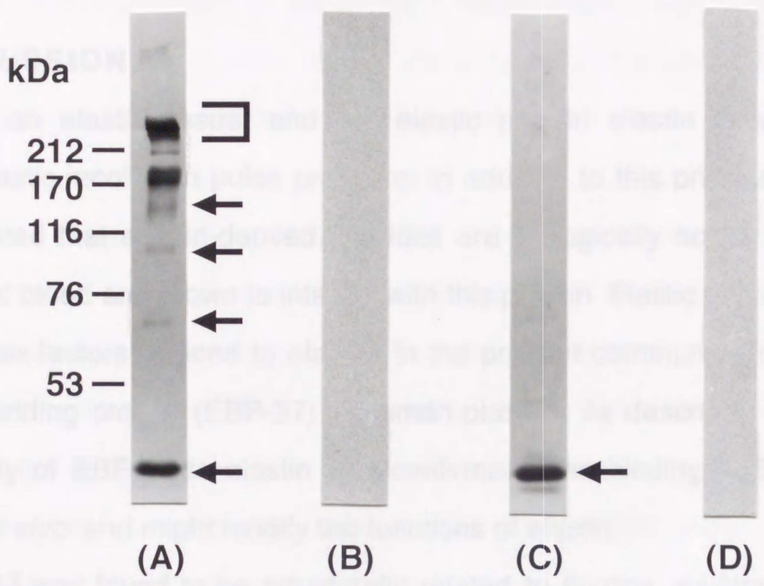


Fig.7. Immunoblot analysis of EBP-37.

Aliquots (10μl) of α-elastin—Sepharose binding fraction, which had an absorbance at 280nm value of 0.3 was subjected to SDS-PAGE under nonreducing (A,B) and reducing (C,D) conditions followed by transfer to PVDF membranes. The membranes were incubated with the EBP-37 antiserum (A,C) and the preimmune serum (B,D) followed by HRP-conjugated secondary antibody. Arrows indicate monomer, dimer, trimer and tetramer forms of EBP-37, and the bracket multimeric forms. The positions of the molecular weight markers are shown on the left.

of EBP-37. Similar patterns of immunoblot analysis were obtained with the recombinant ficolin.

1-4 DISCUSSION

The aorta is an elastic tissue, and the elastic protein elastin provides for energy-dependent elastic recoil with pulse pressure. In addition to this physical function, recent studies indicated that elastin-derived peptides are biologically active (45, 46). Various constituents of blood are known to interact with this protein. Elastic fibers are modified by binding of these factors in blood to elastin. In the present communication, we reported a new elastin-binding protein (EBP-37) in human plasma. As described in chapter 3, the binding activity of EBP-37 to elastin was confirmed. The binding of EBP-37 to elastin might occur *in vivo* and might modify the functions of elastin.

EBP-37 was found to be structurally related to ficolins, multimeric proteins with fibrinogen- and collagen-like domains from porcine uterus membranes (23, 24). Ficolin was originally purified as TGF-β1-binding proteins. Since EBP-37 is very similar to ficolin, this protein may be expected to bind to TGF-β1. If EBP-37 binds to TGF-β1, this protein would play roles in the localization of TGF-β1 in the extracellular matrices of the blood vessel and might be involved in vascular diseases.

Additional or alternative possibility is that EBP-37 might serve as collectin-like proteins (47). There are at least four plasma proteins containing collagenous domains, i.e. C1q, MBP, conglutinin and collectin-43 (48). MBP, conglutinin and collectin-43 are mammalian C-type lectins that bind carbohydrates in a calcium-dependent manner (48). The group III C-type lectins in plasma (48), lung surfactant protein A and D (49, 50) can be classed as collectins. All the molecules belonging to the collectin family consist of multiple polypeptide chains each made up of a short non-collagenous N-terminal segment, followed by a region of collagenous sequence (51). At least C1q, MBP, lung surfactant protein A, conglutinin and collectin-43 bind to the C1q receptor (48). Collectins

have been indicated or suggested to bind to C1q receptors via their collagenous regions (48), C1q, MBP and collectin-43 can activate the classical pathway of complement (48) and lung surfactant protein A (52) can enhance phagocytosis. Since EBP-37 is a plasma protein containing the collagenous sequence, it was expected to bind to C1q receptor.

Recently, a novel human serum lectin with fibrinogen- and collagen- like domains, termed P35, was molecularly cloned and characterized (27). The amino acid sequencing revealed that EBP-37 is identical to P35. P35 was shown to enhance phagocytosis of *Salmonella typhimurium* by polymorphonuclear neutrophils. It was suggested that P35 recognizes GlcNAc residues such as those found in microbial glycoconjugates and complex-type oligosaccharides. However, such oligosaccharides are not present on elastin, and thus the binding of EBP-37 to elastin may occur via a different binding site. Identification of the functional domain that mediates the binding to elastin and comparison with the binding site to GlcNAc may be important for understanding the *in vivo* function of EBP-37.

As described in introduction, a novel corticosteroid-binding protein, termed hucolin, was purified from human plasma (28). Comparison of amino acid sequences indicated that these proteins are identical.

Thus, EBP-37/P35/hucolin appears to function as a human ficolin, and to play important roles with binding activities to diverse substances including elastin, sugar and corticosteroid.

Chapter 2
Identification and Characterization of EBP-37-Like Proteins (Porcine Plasma Ficolins) in Porcine Plasma

2-1 INTRODUCTION

A novel elastin-binding protein (EBP-37) has been identified and purified as described previously. From the viewpoint of comparative biochemistry, the presence of EBP-37-like proteins in various mammalian plasmas was investigated. α- Elastin-Sepharose affinity chromatography of various mammalian plasmas was performed, and each α-elastin-Sepharose-binding fraction was subjected to SDS-PAGE, and then immunoblot analysis was carried out by using anti-EBP-37 antiserum. It was found that a large amount of EBP-37-like proteins (porcine plasma ficolins) are present in the α-elastin-Sepharose-binding fraction of porcine plasma. The EBP-37-like proteins were tentatively termed porcine plasma ficolins (53). The structural and functional properties of porcine plasma ficolins are discussed in this chapter.

2-2 MATERIALS and METHODS

α-Elastin-Sepharose 4B Affinity Chromatography and SDS-PAGE

α-Elastin (25) and α-elastin-Sepharose 4B (ca. 5 mg of α-elastin per ml of hydrated gel) (21) were prepared as described previously. α-Elastin-Sepharose 4B affinity chromatography of various mammalian plasmas was performed at 25°C as described previously (21, 22). The α-elastin-Sepharose-binding fraction was subjected to SDS-PAGE (8% or 10% polyacrylamide gel) as described in chapter 1. Gels were stained with CBB R-250 (Merck).

Amino Acid Sequencing of a 40 kDa Protein in α-Elastin-Binding Fraction of Porcine Plasma

Electroblotting of the α-elastin-binding proteins of porcine plasma from SDS-gel (10% polyacrylamide gel, nonreducing conditions) onto a polyvinylidene difluoride (PVDF) membrane (Immobilon-P, Millipore) was performed as described (36). The membrane was stained with Coomassie Brilliant Blue R-250. The peptide bands were then excised. Internal peptide sequences of the 40 kDa protein were obtained using the peptide mapping technique of Cleveland *et al.* as described in chapter 1. The 40 kDa protein was digested with *Staphylococcus aureus* V8 protease (Boehringer Mannheim). As described in chapter 1, the amino acid sequences were determined by use of a pulse-liquid-phase amino acid sequencer (Model 477A protein sequencer, Applied Biosystems).

Immunoblot Analysis of Porcine Plasma Ficolins

The α-elastin-Sepharose binding fraction of porcine plasma was mixed with SDS-sample buffer, boiled at 95°C for 4 min, and subjected to SDS-PAGE (8% polyacrylamide gel) under nonreducing or reducing conditions. After electrophoresis, the proteins were electrophoretically transferred to PVDF membranes, as described previously. Immunostaining of porcine plasma ficolins was performed with an anti-EBP-37 antiserum as previously described (22) or an antiserum denoted AN1801, reactive with both ficolin-α and -β, that was raised against a synthetic peptide (24). Immunostainings of plasma ficolin-like proteins in α-elastin-Sepharose binding fractions of various mammalian plasma were performed with anti-EBP-37 antiserum. The procedure of staining is described in chapter 1.

2-3 RESULTS

2-3-1 *Identification of EBP-37-Like Proteins (Ficolin-Like Proteins) in the α-Elastin-Binding Fraction of Porcine Plasma*

To identify α-elastin binding proteins in porcine plasma, plasma was passed through a Sepharose 4B column and then applied to an α-elastin-Sepharose 4B column at 25°C. The α-elastin-Sepharose-binding fraction was subjected to SDS-PAGE and analyzed by staining with CBB R-250. Figure 8 shows the migration patterns of these proteins in SDS-PAGE (10% polyacrylamide gel) under reducing (Fig.8 A) and nonreducing (Fig.8 B) conditions. Several bands with different molecular sizes, including 27, 37-40, 54, 62 and 76-88-kDa, were observed under reducing conditions, and two major bands with sizes of 37-40 and 250-kDa were seen under nonreducing conditions. The doublet band of 40-kDa and 37-kDa was found only in the eluate from the α-elastin-Sepharose 4B column, but not from the plain Sepharose 4B column under reducing and nonreducing conditions (data not shown). The migration profile of the doublet band of 40-kDa and 37-kDa was similar to that observed in immunoprecipitates of recombinant porcine ficolin-α (24). In the case of human plasma, only a single band with an apparent molecular mass of 37-kDa was observed (22). As described below, sequencing studies indicated that the upper 40-kDa protein of the doublet band under nonreducing conditions is identical or closely related to porcine ficolin-α. However, two-dimensional gel electrophoresis revealed that several isoelectric variants appear to exist in unequal amounts in the 40 kDa and 37 kDa proteins (data not shown).

2-3-2 *Immunoblot Analysis of Porcine Plasma Ficolins and Distribution of Porcine Plasma Ficolins-Like Proteins in α-Elastin-Binding Fractions of Various Mammalian Plasmas*

The α-elastin-Sepharose binding fraction of porcine plasma was subjected to SDS-PAGE under nonreducing and reducing conditions, and then immunoblot analysis was

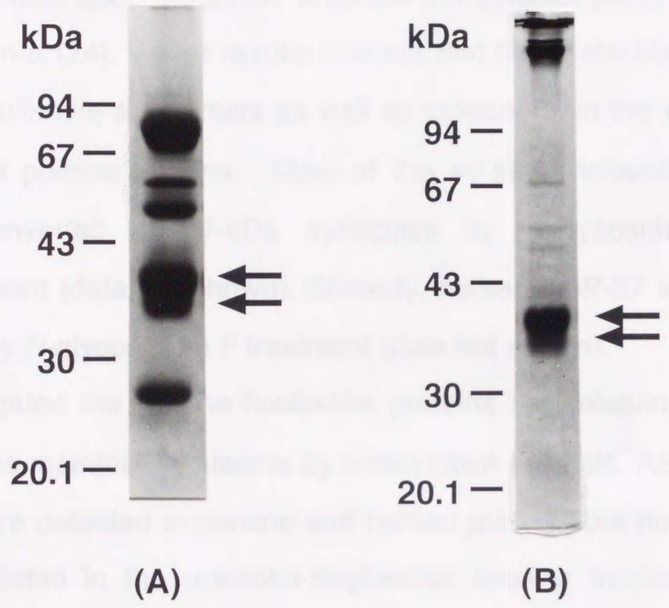


Fig. 8 SDS-PAGE analysis of α-elastin-Sepharose binding proteins of porcine plasma.

Aliquots (15 μl) of α-elastin-Sepharose binding fraction, which had an absorbance at 280 nm of 1.2, were subjected to SDS-PAGE (10% polyacrylamide gel) under reducing (A) and nonreducing (B) conditions. The gels were stained with Coomassie Brilliant Blue R-250. The arrows indicate the 40 kDa and 37 kDa proteins. The positions of the molecular weight markers are shown on the left.

carried out by using anti-EBP-37 antiserum (22) (Fig.9 (A,B)) and AN1801 antiserum (24) (Fig.9 (C,D)). As shown in Fig.9 (A-D), similar patterns were obtained with both antisera. High-molecular-weight components of more than 200 kDa and 3 different molecules with sizes of about 75, 40 and 37-kDa reacted with the anti-EBP-37 antiserum and AN1801 under nonreducing conditions; the high-molecular components were all converted to 40 and 37-kDa molecules upon reduction. A similar immunoblot pattern was obtained with recombinant ficolin-α (24). These results indicate that the ficolin-like molecules exist as disulfide-linked multimers and dimers as well as monomers in the α-elastin-Sepharose binding fraction of porcine plasma. Most of the 40-kDa molecules found in porcine plasma were converted to 37-kDa molecules by *N*-glycosidase F (Boehringer Mannheim) treatment (data not shown). Similarly, human EBP-37 was shifted from 37-kDa to 34.5-kDa by *N*-glycosidase F treatment (data not shown).

We investigated the plasma ficolin-like proteins in α-elastin-Sepharose binding fractions of various mammalian plasma by immunoblot analysis. As shown in Fig.9 (E), strong signals were detected in porcine and human plasma, but no signal of ficolin-like proteins was detected in the α-elastin-Sepharose binding fractions of plasma from sheep, rat, bovine and goat.

2-3-3 *Amino Acid Sequencing of the 40-kDa and 37-kDa Proteins in α-Elastin-Binding Fraction of Porcine Plasma*

Determination of the amino-terminal amino acid sequence of the 40 kDa protein revealed that it is identical to that of porcine ficolin-α (Fig.10 (A,B), N-term.) (53). The internal amino acid sequences of the 40-kDa protein (V1, V2) were determined after V8 protease digestion. The sequence of peptide V1 was also identical to that of ficolin-α except the 4th amino acid residue, which was Pro instead of Thr as reported in a previous study (24). There are at least two possible reasons for this difference: one is that the sequence of peptide V1 may come from a ficolin-like peptide chain other than ficolin-α and -β, and the other is that this difference may reflect protein polymorphism or

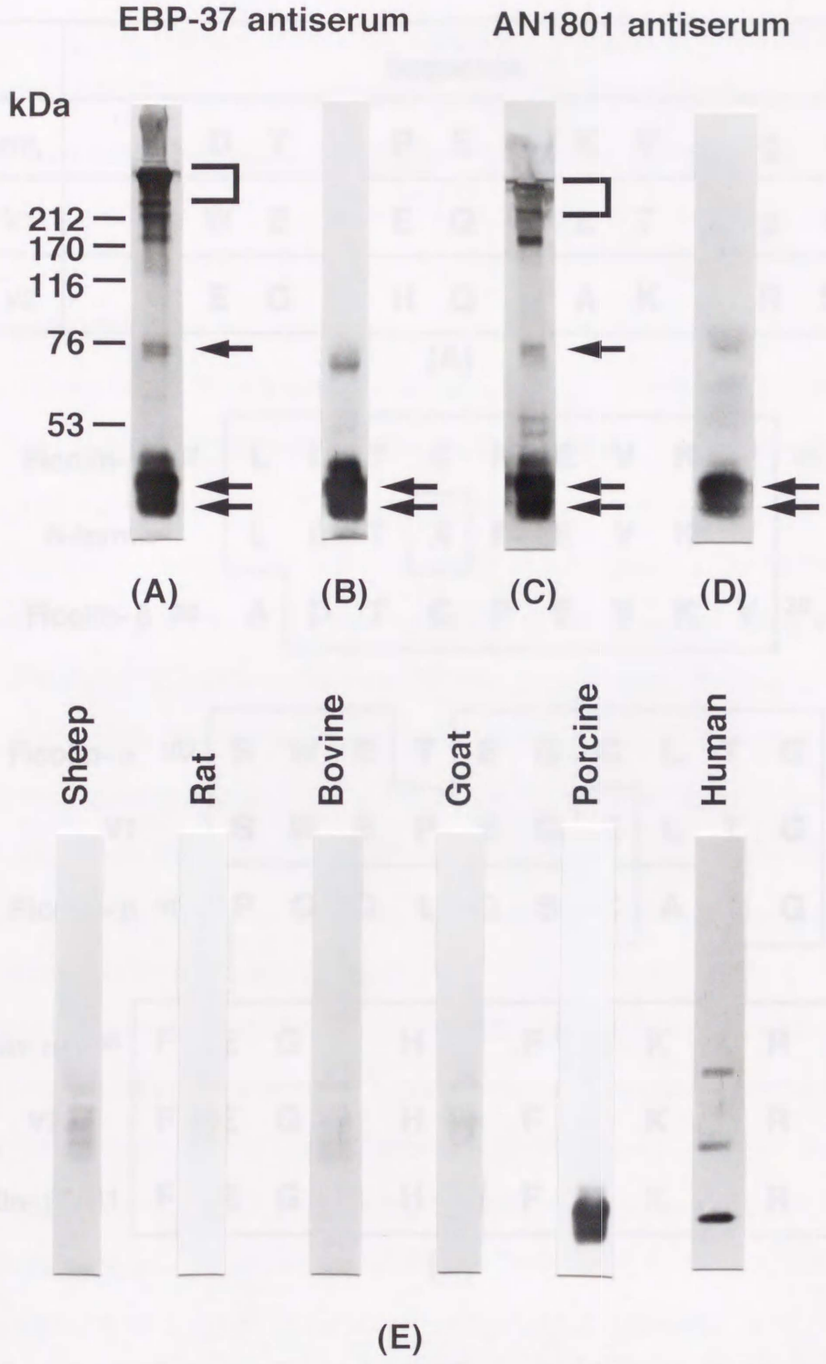
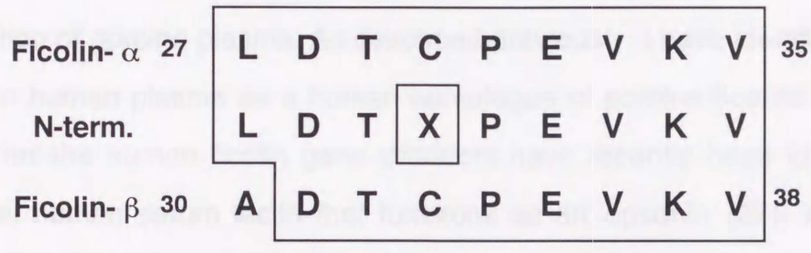
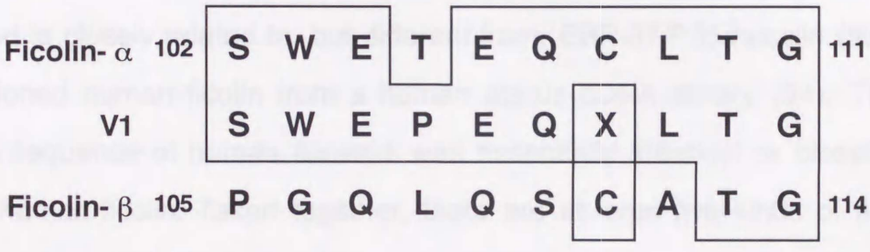


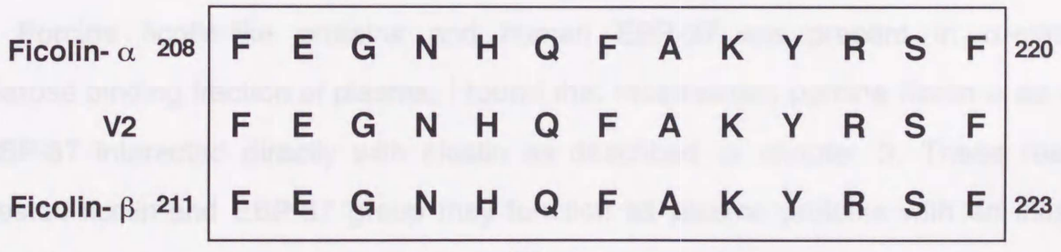
Fig. 9 Immunoblot analysis of porcine plasma ficolins and α-elastin-Sepharose binding fractions of various mammalian plasma. Alquots (7 μl) of α-elastin-Sepharose binding fraction of porcine plasma, which had an absorbance at 280nm of 0.5, were subjected to SDS-PAGE (8% polyacrylamide gel) under nonreducing (A, C) and reducing (B, D) conditions followed by transfer to PVDF membranes. The membranes were incubated with the EBP-37 antiserum (A, B) and AN1801 antiserum (C, D) and immunoreactive proteins were visualized using horseradish peroxidase-conjugated secondary antibody. The arrows indicate monomer and dimer of ficolins, and the bracket multimeric forms. The positions of the molecular weight markers are shown on the left. Immunoblot analyses of α-elastin-Sepharose binding fractions of various mammalian plasma were performed with the same method under reducing conditions by using the EBP-37 antiserum (E).

	Sequence
N-term.	L D T X P E V K V v g l e
V1	S W E P E Q X L T G p r
V2	F E G N H Q F A K Y R S F

(A)







(B)

Fig. 10 Amino acid sequencing of the 40 kDa protein from α-elastin binding fraction of porcine plasma and sequence comparison between the 40 kDa protein and porcine ficolins.

(A) Amino-terminal amino acid sequence (N-term.), amino acid sequences of V8 protease digestion fragment 1 (V1) and 2 (V2) of the 40 kDa protein are shown. X denotes an unidentified residue. The sequence shown in small letter code involves some ambiguity.

(B) Comparison of the amino acid sequences of ficolin-α and -β with those obtained from N-term., V1 and V2. Identical amino acid residues are boxed.

difference among the porcine race. The sequence of peptide V2 was identical to both ficolin-α and -β (24). These results strongly suggested that the 40 kDa protein identified in the α-elastin binding fraction is porcine ficolin-α and/or closely related molecules.

2-4 DISCUSSION

In this study, it was found that ficolin-like proteins are present in an α-elastin-Sepharose binding fraction of porcine plasma. As described previously, I have identified and purified EBP-37 from human plasma as a human homologue of porcine ficolins (22). Two other candidates for the human ficolin gene products have recently been identified. One is P35, a novel human serum lectin that functions as an opsonin (27). As described in chapter 1, EBP-37, P35, and hucolin are identical. As described below, human ficolin-1 in this report is closely related to, but different from, EBP-37/P35/hucolin (53). Recently, Lu *et al.* cloned human ficolin from a human uterus cDNA library (54). The deduced amino acid sequence of human ficolin-1 was essentially identical or closely related to that of the human ficolin. Taken together, there are at least two kinds of ficolin-related proteins in human, as in the case of pig.

Porcine ficolin-like proteins and human EBP-37 are present in α-elastin-Sepharose binding fraction of plasma. I found that recombinant porcine ficolin-α as well as EBP-37 interacted directly with elastin as described in chapter 3. These results suggested ficolin and EBP-37 group may function as plasma proteins with an elastin-binding activity. We could not detect ficolin-like proteins in α-elastin binding fractions of sheep, rat, bovine and goat plasma by immunoblot analysis. The reasons for the negative results are currently unknown; however, plasma ficolin-like proteins might exist in significant amounts only in porcine and human plasma, as in the case of conglutinin, which is a serum lectin that is present in significant amounts only in members of the *Bovidae* (55). Alternatively, anti-EBP-37 antiserum might have species specificities in its epitope recognition.

Another important function of porcine plasms ficolins has been suggested by the recent cloning and characterization of P35 as a human serum Ca^{2+}-dependent lectin (27). P35 was shown to enhance phagocytosis of *Salmonella typhimurium* strain TV119 by polymorphonuclear neutrophils. It was suggested that P35 recognizes a large number of nonreducing terminal GlcNAc residues exposed on the surface and functios as an opsonin. Similarly, it was suggested porcine plasma ficolins may also function as an opsonin.

Immunoblot analyses of porcine plasma ficolins revealed that the high-molecular components were all converted to 40 and 37-kDa molecules upon reduction. Similar patterns of immunoblot analysis were obtained with recombinant ficolin. Similarly, the high-molecular components of EBP-37 were all converted to 37-kDa molecules upon reduction. However, the natural material of ficolin from porcine uterus membranes contained very little of the monomeric from under reducing conditions (23). One difference between the natural ficolin, recombinant ficolins, plasma ficolins, and EBP-37 appears to be resistance to reducing agents. The fact that ficolin from the natural source is partially resistant to reducing agents suggests that a difference in cross-links may exist between each chain. Microfibril-associated glycoprotein (MAGP) (56), fibronectin, vitronectin, and collagen typeIII have all been shown to be specific substrates for transglutaminase (57). The natural material of ficolin might be the substrate for transglutaminase-catalyzed cross-linking. The cross-links might be produced in the natural material. in addition to disulfide bonds. There might be matrix associated ficolins and plasma ficolins. EBP-37 and porcine ficolins might be present as matrix associated materials and secreted materials in blood.

There could be microheterogeneity in porcine plasma ficolin-like proteins and EBP-37, based on the results of two-dimensional gel electrophoresis (data not shown). It is possible that the alteration of a peptide portion and the post-translational processing of ficolins alter the structure of the whole molecule. In order to understand the *in vivo* functions of the ficolin and EBP-37 group, it will be important to analyze the related gene

products and the mechanism of polymerization, and to investigate the localization of the molecules in the extracellular matrix.

Chapter 3
Bindings of EBP-37 and Recombiant Ficolin-α to Elastin

3-1 INTRODUCTION

As already mentioned in "Chapter 1 and 2", EBP-37 and porcine plasma ficolins were identified by using α-elastin-Sepharose affinity chromatography.

In order to confirm the direct interaction of EBP-37 and porcine plasma ficolins with elastin, binding assays were performed by using purified EBP-37 and recombinant porcine ficolin-α. Detailed experiments of the associations of EBP-37 with other matrix molecules *in situ* are in progress. This chapter will concentrate on studies of the interactions of purified EBP-37 and recombinant porcine ficolin-α with elastin *in vitro*.

3-2 MATERIALS AND METHODS

Assay for Binding of EBP-37 to α-Elastin on the Nitrocellulose Membrane

Binding assay was performed by dot blot analysis using the Biodot and nitrocellulose membranes. As described in chapter 1, extracted 37-kDa protein from SDS-gels was used as the purified EBP-37. A sample (3μg) of α-elastin was dot blotted on a nitrocellulose membrane. The coating of α-elastin in the dot blots were confirmed by amido black staining. The membrane was inactivated with 1%(w/v) bovine serum albumin (BSA) in PBS. After being washed with PBS three times, the dots of the membrane were incubated and soaked with the respective quantity of EBP-37 in PBS. Control blots were incubated with buffer only. After incubation, they were washed with PBS three times, and then immunostained with the EBP-37 antiserum or preimmune serum diluted 1/2000 in 1%(w/v) BSA inPBS. The blots were then visualized with HRP-

conjugated goat anti-rabbit IgG diluted 1/2000 in the same solution, 0.05% 3,3'-diaminobenzidine, 0.045% H_2O_2 and 0.84mM $CoCl_2$ in 10mM Tris-HCl, pH7.5.

Stepwise Elution of α-Elastin-Sepharose Binding Proteins of Human Plasma

In order to investigate the mode of interaction between EBP-37 and α-elastin, stepwise elution of EBP-37 from α-elastin-Sepharose was perfomed with various solvents. α-Elastin-Sepharose binding proteins were eluted with 1M NaCl-0.05M Tris-HCl (pH7.4 at 25°C), 1M KBr-0.05M sodium acetate buffer (pH5.3 at 25°C) and 8M urea-0.05M Tris-HCl (pH7.4 at 25°C).

Preparation of Recombinant Porcine Ficolin-α

The ficolin-α-transfected porcine aortic endothelial cells (PAE) cells were kindly provided by Dr. Hidenori Ichijo (The Cancer Institute). Construction of expression plasmid and cell transfection are as follows (24).

A stable transfection plasmid pcnFCα was constructed by subcloning the ficolin-α cDNA insert into the vector pcDNAI neo (In Vitrogen). For stable transfection of the ficolin-α cDNA, the PEA cell line (58), maintained in Ham's F-12 medium supplemented with 10% fetal calf serum, was transfected with pcnFCα using electroporation, as described (24). Selection of transfected cells was performed in the presence of G418 (Genectin, GIBCO/BRL). Resistant cell colonies were examined for the expression of ficolin-α by metabolic labeling and immunoprecipitation, and studied by immunoblotting.

Metabolic Labeling

Metabolic labeling of the ficolin-α-transfected PAE cells was performed for 12 h with [^{35}S]methionine and cysteine after confluence. After labeling, the medium was collected and centrifuged for 5 min at 19,000 xg, and the supernatant was used for the binding experiment.

Assay for Binding of Recombinant Porcine Ficolin-α to Insoluble Elastin, α-Elastin-Sepharose and Gelatin-Sepharose

Autoclaved insoluble elastin (from 4 mg of dried powder, Sigma), α-elastin-Sepharose 4B beads (20 μl of wet gel, ca. 5 mg of α-elastin per ml of hydrated gel) (21), gelatin-Sepharose 4B beads (20 μl of wet gel, ca. 6 mg of gelatin per ml of hydrated gel, Pharmacia Biotech) and Sepharose 4B beads (20 μl of wet gel, Pharmacia Biotech) were incubated with 1 ml of the radio-labeled medium for 12 h at 4°C. Washing was performed four times with 50 mM Tris-HCl, pH 7.4, containing 1 M NaCl and 1% Triton X-100 and once with distilled water. Bound materials were eluted by boiling for 5 min in SDS-sample buffer (100 mM Tris-HCl, pH 8.8, 0.01% bromphenol blue, 36% glycerol, 4% SDS) and analyzed by a 5 to 15% gradient SDS-PAGE under non-reducing conditions. Gels were fixed and subjected to fluorography.

3-3 RESULTS

3-3-1 *Direct Binding of EBP-37 to α-Elastin*

In order to examine whether EBP-37 interacts with α-elastin directly or it binds to the α-elastin—Sepharose column via the interaction with other proteins such as Igs, the binding of isolated EBP-37 to α-elastin was studied by using the EBP-37 antiserum. α-Elastin was immobilized on nitrocellulose membranes. The membrane was incubated with EBP-37 and bound EBP-37 was detected by immunostaining with the EBP-37 antiserum. As shown in Fig.11 (A—D), EBP-37 was shown to bind to the immobilized α-elastin according as the quantity of EBP-37. Control blots were not stained (Fig.11 F), and EBP-37 did not react with the preimmune serum (Fig.11 G).

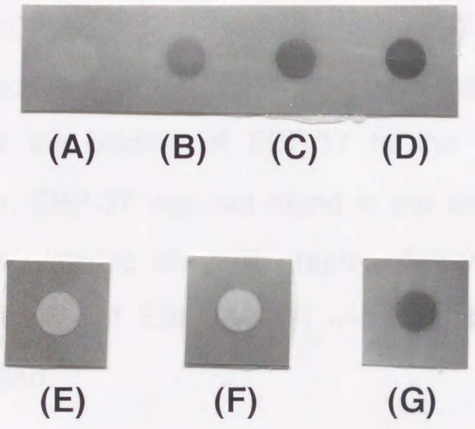


Fig.11. Binding of EBP-37 to α-elastin on membranes.
α-Elastin was dot blotted onto nitrocellulose membranes. 30ng of EBP-37 was dot blotted onto a nitrocellulose membrane as the positive control (G). Blots were incubated with 10 (A), 30 (B), 60 (C), 120 (D) ng of EBP-37. Dot blot (E) was incubated with buffer alone. Dot blot (F) was incubated with the preimmune serum as the antibody followed by HRP-conjugated secondary antibody. All other dot blots (A-E, G) were incubated with the EBP-37 antiserum followed by HRP-conjugated secondary antibody.

3-3-2 *Mode of Interaction of EBP-37 with α-Elastin*

To investigate the manner of the interaction between EBP-37 and α-elastin, stepwise elution of EBP-37 from the α-elastin-Sepharose column was performed. SDS-PAGE(12.5% polyacrylamide gel) under nonreducing conditions revealed EBP-37 was present in the 1M KBr(pH5.3) eluate (Fig.12 B) and the 8M urea eluate (Fig.12 C), but not present in the 1M NaCl (Fig.12 A). Furthermore, EBP-37 was eluted from the column with 1M KI(pH7.4), but not with 1M NaCl(pH5.3) or 1M KBr(pH7.4) (data not shown). The results indicate that the binding of EBP-37 to the α-elastin involves hydrophobic interaction. However, EBP-37 was not found in the eluates in phenyl-Sepharose and butyl-Sepharose hydrophobic chromatography of human plasma (data not shown). Therefore, the interaction of EBP-37 with α-elastin does not seem to be a simple hydrophobic interaction.

3-3-3 *Elastin-Binding Activity of Recombinant Ficolin-α*

As described previously, EBP-37 from human plasma bound most efficiently to α-elastin-resin, compared with gelatin (derived from type I collagen), type II collagen, fibronectin and fibrinogen-resin (22). In order to study whether porcine ficolin-α interacts with elastin, binding assay was performed using metabolically labeled porcine ficolin-α which was produced from the cells transfected with the ficolin-α cDNA. As shown in Fig.13, recombinant porcine ficolin-α bound to insoluble elastin as well as α-elastin-Sepharose, as a 37-40 kDa monomer, 70-80 kDa dimer and several higher molecular weight multimers. It also bound to gelatin-Sepharose, but the amount was much less than that recovered from the insoluble elastin or α-elastin-Sepharose. Ficolin-α did not bind to the plain Sepharose beads. These results further suggested a direct interaction of recombinant porcine ficolin-α with elastin. Furthermore, it was found that an efficient association of partially purified EBP-37 from human plasma with α- and κ-elastin, and laminin, and to a lesser extent with gelatin, but only marginally with types I, II, III, and IV collagen, fibronectin and vitronectin by using an ELISA method (data not shown, but the

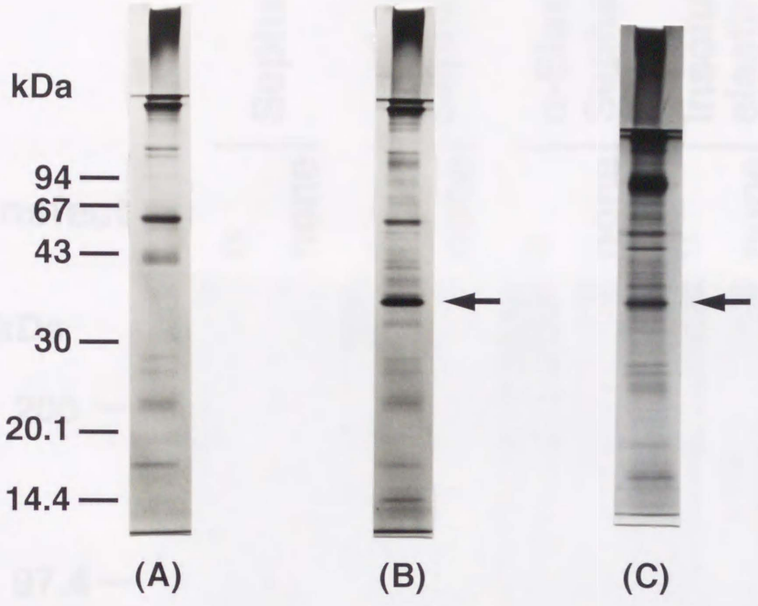


Fig.12. Elution of EBP-37 from the α-elastin—Sepharose column with various soluvents.

Human plasma was applied to the α-elastin—Sepharose column and the column was successively eluted with 1M NaCl(pH7.4), 1M KBr(pH5.3) and 8M urea(pH7.4). A portion of the eluate was subjected to SDS-PAGE under nonreducing conditions. Proteins were visualized by silver staining. Lane(A), 1M NaCl(pH7.4) eluate; Lane(B), 1M KBr(pH5.3) eluate; Lane(C), 8M urea(pH7.4) eluate. The positions of the molecular weight markers are shown on the left. The arrow indicates the 37-kDa protein.

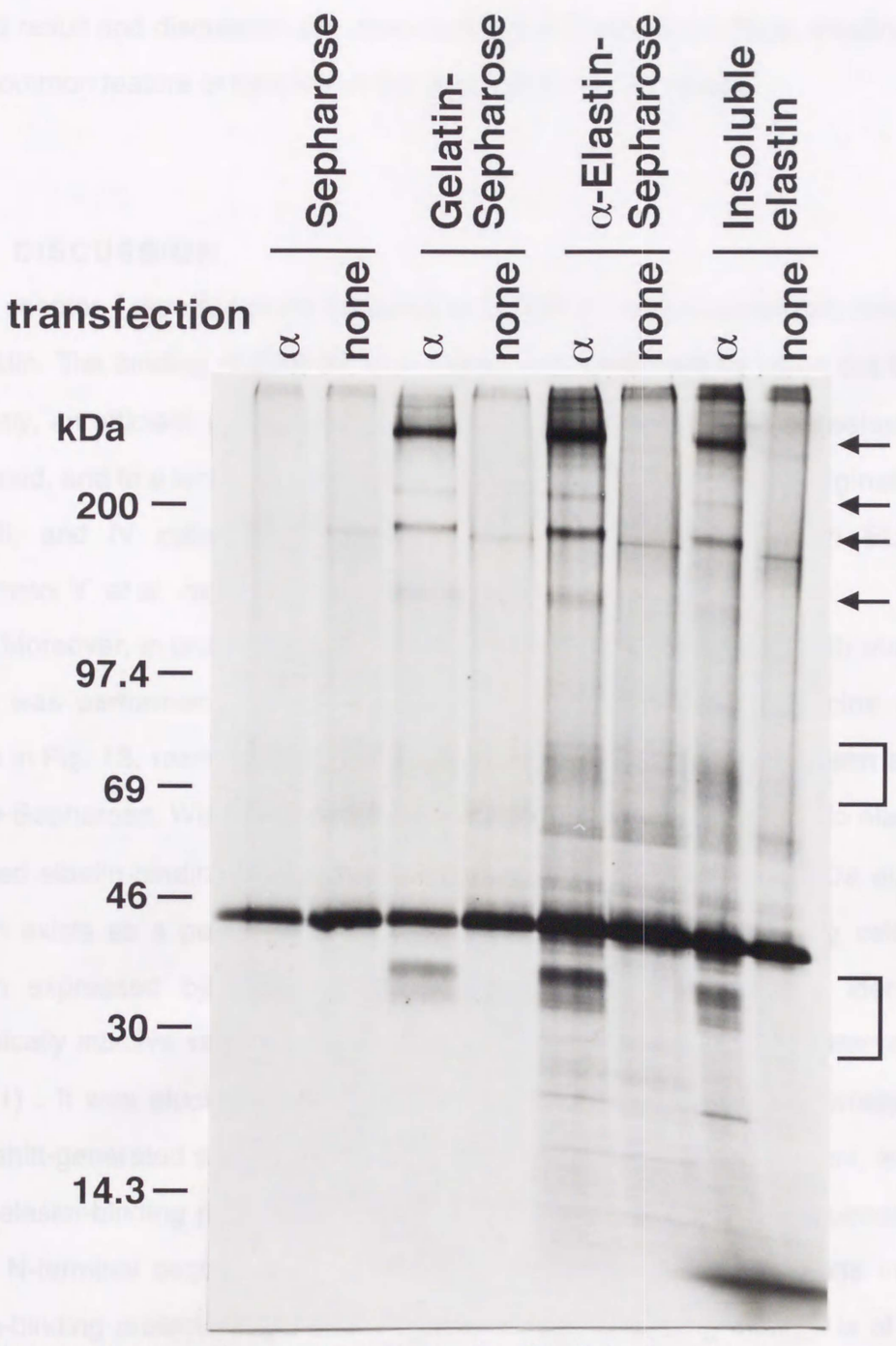


Fig. 13 Binding of recombinant porcine ficolin-α to elastin.

Control PAE cells (none) and PAE cells transfected with ficolin-α (α) were metabolically labeled with [^{35}S]methionine and cysteine. The media were then subjected to the binding assay (see text). Specific bands present in the medium of transfected PAE cells are indicated by arrows and brackets. The migration distances of molecular weight markers are indicated on the left.

related result and discussion are described in 3-4 Discussion.) Thus, elastin-binding may be a common feature or function of the ficolin and EBP-37 group.

3-4 DISCUSSION

In this chapter, I reported direct interactions of EBP-37 and recombinant porcine ficolin-α to elastin. The binding of EBP-37 to α-elastin was confirmed by using dot blot analysis. Recently, an efficient association of partially purified EBP-37 with tropoelastin was also confirmed, and to a lesser extent with laminin and gelatin, but only marginally with types I, II, III, and IV collagen, fibronectin, and vitronectin by using an ELISA method (Fukumoto, Y. *et al.* manuscript in preparation).

Moreover, in order to study whether porcine ficolin-α interacts with elastin, binding assay was performed using metabolically labeled recombinant porcine ficolin-α. As shown in Fig. 13, recombinant porcine ficolin-α bound to insoluble elastin as well as α-elastin-Sepharose. We speculated the binding site of porcine ficolin-α to elastin from the reported elastin-binding sequences (22). It was reported that a 67-kDa elastin-binding protein exists as a peripheral membrane protein of elastin-producing cells (59). This protein expressed by sheep aorta smooth muscle cells may be identical to the catalytically inactive variant of human β-galactosidase produced by alternative splicing (60, 61) . It was elucidated that the VVGSPSAQDEASPL domain, corresponding to a frameshift-generated sequence unique to the alternatively spliced variant, is responsible for its elastin-binding properties (61, 62). In addition, homologous sequences are found in the N-terminal sequences of several serine elastases. These results indicated that elastin-binding proteins might share a common ligand-binding motif. It is of interest that an elastin-binding motif of similar sequence is also found in ficolin-α,β (Fig.14). It was reported that 67-kDa elastin-binding protein binds to the VGVAPG hydrophobic domains on elastin (59) and blocking of VGVAPG sequences on elastin by a monoclonal antibody inhibits degradation of elastin by elastases (62). We speculate that the

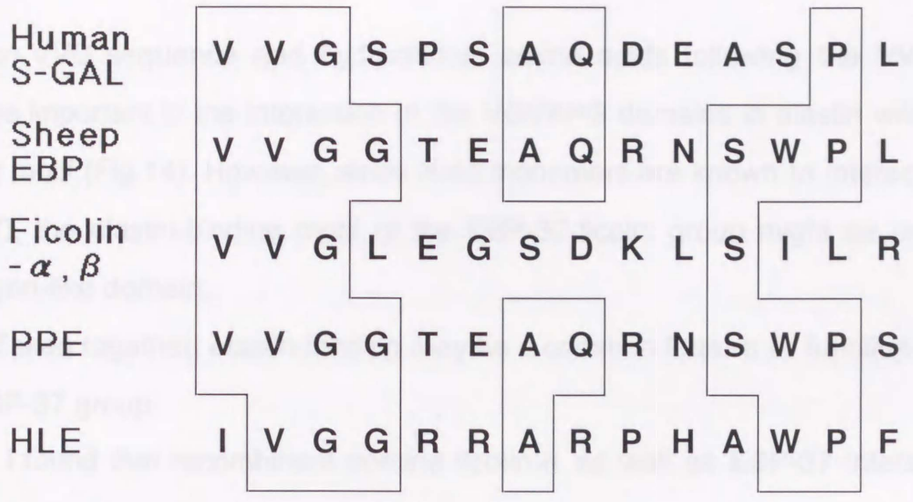


Fig.14. Comparison of the partial amino acd sequence of ficolin-α,β with the partial amino acid sequences of human S-GAL, sheep EBP, PPE and HLE.
The elastin-binding sequence from the spliced variant of human β-galactosidase (human S-GAL) (60), the sequence determined for the CNBr fragment of purified sheep elastin-binding protein (EBP) (61), N-terminal sequences of porcine pancreatic elastase (PPE) (63) and human leukocyte elastase (HLE) (64) are compared with the sequence 35—48 in ficolin-α and 38—51 in ficolin-β (24). Amino acids identical in more than two members are boxed.

common VVG sequence and hydrophobic amino acids following the VVG sequence might be important in the interaction of the VGVAPG domains in elastin with the elastin-binding motif (Fig.14). However, since fibrin monomers are known to interact with elastin (16, 17), the elastin-binding motif of the EBP-37-ficolin group might be present in the fibrinogen-like domain.

Taken together, elastin-binding may be a common feature or function of the ficolin and EBP-37 group.

I found that recombinant porcine ficolin-α as well as EBP-37 interacted directly with elastin, which is a core protein of elastic fibers together with surrounding microfibrils, a complex of glycoproteins (65). Major components of the elastin-associated microfibrils are large (350-kDa), rod-like glycoproteins called fibrillins (11) and a small (31-kDa), elastin-binding glycoprotein called microfibril-associated glycoprotein (MAGP) (10). As described in chapter 4, a human microfibril-associated glycoprotein (MFAP4) which shows 51 to 52% amino acid sequence identity to porcine ficolins in their fibrinogen-like domain is also a microfibril component. It was reported that microfibril-associated glycoprotein (MAGP) is an integral component of microfibrillar structures that play a critical role in the organization of elastic fibers in the ECM, and MAGP specifically binds to tropoelastin. Two fibrillins (fibrillin 1 and fibrillin 2) have been cloned (66, 67, 68) and linked to the congenital disorders of Marfan syndrome. Marfan syndrome is characterized by major abnormalities of the skeletal and cardiovascular systems and is often associated with ocular defects. It is evident that fibrillins and thus microfibrils are essential for the normal development, morphology, and function of a wide range of tissues. The overall structures of fibrillins are similar to those of latent TGF-β binding proteins (LTBP) (66, 69), which bind to the N-terminal precursor parts of TGF-β via a disulfide bridge. Among the three isoforms of LTBPs, LTBP-1 and -2 have been shown to be components of microfibrils (70). Interestingly, bovine LTBP-2 was immunolocalized to the elastin-associated microfibrils (71). In view of the fact that ficolins were identified and

purified as TGF-β1 binding proteins and possibly colocalize with LTBPs, they may modulate the activity of TGF-β *in vivo*.

Moreover, recent evidence has suggested that TGF-β in blood may regulate atherogenesis (72) and it was proposed that TGF-β is a key inhibitor of atherosclerosis (73). To understand the functions of porcine ficolin-like proteins and EBP-37 in blood, it is necessary to investigate the interactions of porcine ficolin-like proteins and EBP-37 with TGF-β.

Chapter 4

Molecular Cloning of Human Ficolin-1 and Expression of Human Ficolin-1 in Human Tissues

4-1 INTRODUCTION

As described in chapter 1, EBP-37 purified as a 37-kDa component of newly identified elastin-binding proteins from human plasma. To obtain a cDNA that encode EBP-37, cDNA cloning was performed.

Recently, a novel human serum lectin with fibrinogen- and collagen-like domains, termed P35, was molecularly cloned and characterized (27). In progress of cDNA cloning, I found that EBP-37 and P35 are identical from comparison of amino acid sequences of these proteins. However, I obtained a cDNA that is closely related to, but different from, EBP-37/P35/hucolin by screening a human uterus cDNA library with porcine ficolin-α cDNA as a probe. Since the cDNA encodes a protein which is equally similar to porcine ficolin-α and -β, this clone was termed human ficolin-1. Northern blot analysis of various human tissues revealed that human ficolin-1 mRNA is highly expressed in peripheral blood leukocytes. Taken together, these observations suggested that there are at least two ficolin-related proteins in both pig and human, which may have important roles as plasma proteins.

4-2 MATERIALS AND METHODS

Screening a Phage Library

The human uterus λgt10 cDNA library (Clontech) with $2x10^6$ clones was plated and lifted onto nitrocellulose filters (Hybond-C extra, Amersham), and immobilized by baking for 1h at 80°C. The duplicate filters were probed with ^{32}P-labeled porcine ficolin-α cDNA at

37°C overnight in the hybridization buffer containing 50mM $NaHPO_4$, pH6.5, 50% formamide, 5 X SSC (1 X SSC is 15mM sodium citrate, 150mM NaCl), 0.1% SDS, 5 X Denhardt's solution, and 100μg/ml of salmon sperm DNA. Porcine ficolin-α cDNA was kindly provided by Dr. Hidenori Ichijo (The Cancer Institute). ^{32}P-labeling of the porcine ficolin-α cDNA was done with a Ready-To-Go DNA Labeling Kit (Pharmacia Biotech). The filters were washed two times with 2 X SSC, 0.1% SDS at room temperature for 15 min, followed by 0.5 X SSC, 0.1% SDS at 50°C for 10 min two times. The primary screening yield 33 positive clones. In the secondary screening, 26 positive clones were detected. Inserts of the positive clones were amplified by PCR using λgt10 forward and reverse primers, and subcloned into pGEM-T vector (Promega). Nucleotide sequencing was performed with a Sequenase Version 2.0 DNA sequencing kit (United States Biochemical).

Anchored PCR

To obtain a full-length cDNA clone of human counterparts of porcine ficolins, anchored PCR was performed by Taq DNA polymerase using the human uterus cDNA library as a template. λgt10 reverse primer, AS15S-1 primer (5'-CCCCCGGTCTAGCAGGTCCTT-3'), and AS15S-2 primer (5'-TGGCTGGGGAAATGGGGTGAC-3') were used as PCR primers. PCR was performed for 25 cycles of 94°C (45 sec), 48°C or 55°C (45 sec) and 72°C (90 sec). The PCR products were subcloned into pGEM-T vector and were sequenced as described above.

Computer Alignment

Comparison of the overall amino acid sequence was performed by using the Clustal computer alignment program (74) of the Lasergene program (DNASTAR).

Northern Blot Analysis

Hybridizations of human multiple tissue northern (MTN) blot and MTN blot II (Clontech) were performed in the hybridization buffer at 37°C overnight with ^{32}P-labeled human ficolin-1 cDNA fragment (nucleotides 262-1194) as a probe. The blots were sequentially washed in 2 X SSC, 0.1% SDS at room temperature for 30 min, 0.5 X SSC, 0.1% SDSat 50°C for 80 min, and 0.5 X SSC, 0.1% SDSat 65°C for 30 min, and then subjected to autoradiography.

4-3 RESULTS

4-3-1 *Cloning of a Human Ficolin cDNA*

A human uterus cDNA library was screened with the porcine ficolin-α cDNA as a probe. The primary screening yielded 33 positive clones. Three of the longest clones, denoted 15S7, 8S and 11S, were sequenced entirely. The sequence of 15S7 clone was found to encode an incomplete amino acid sequence of human ficolin-1 (Fig.15, cDNA sequence corresponding to nucleotides 262-1194). The sequences of other clones (8S, 11S) were found to encode a human microfibril-associated glycoprotein (MFAP4); the gene for MFAP4 is deleted in Smith-Magenis syndrome (12). To clone the complete amino-terminal fragment of human ficolin-1, anchored PCR was performed. The largest PCR product was found to encode the amino-terminal part of human ficolin-1 (Fig.15, cDNA sequence corresponding to 1-369), and thereby the complete human ficolin-1 cDNA was obtained (Fig.15). A 1194 bp nucleotide sequence has an ATG start codon at position 16, followed by a 957 bp open reading frame which encodes a peptide with 319 amino acids. A hydrophobic signal sequence was observed after the ATG start codon; the 3'-untranslated sequence contained a poly(A) tail.

The primary structure of human ficolin-1 has two distinct features, *i.e.,* a collagen-like domain following a short N-terminal signal peptide sequence according to the rules

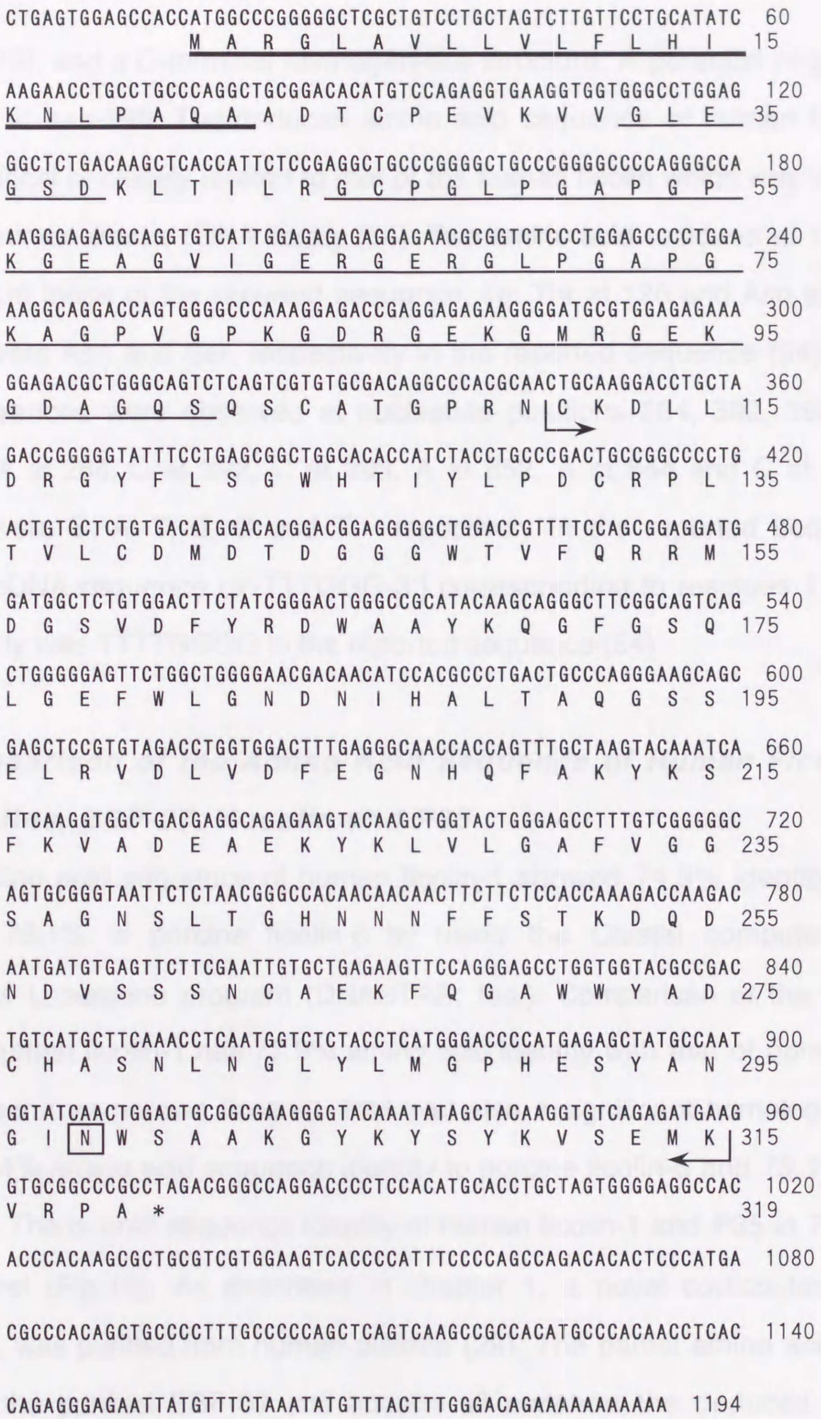


Fig.15 Nucleotide and deduced amino acid sequences of human ficolin-1 cDNA.
The deduced amino acid sequence of human ficolin-1 cDNA is shown below the nucleotide sequence. The putative signal sequence according to the rules of von Heijne is underlined with a thick line. The collagen-like domain is underlined. The fibrinogen-like domain (arrows) and a potential *N*-linked glycosylation site (box) are shown.

of von Heijne (75), and a C-terminal fibrinogen-like structure. A potential *N*-glycosylation site was found at Asn-298. The deduced amino acid sequence of human ficolin-1 was essentially identical or closely related to that of the human ficolin which was very recently cloned from a human uterus cDNA library (54). Two amino acid residues at 126 and 280 are different from those of the reported sequence, *i.e.* Thr at 126 and Asn at 280 in the present study were Asn and Ser, respectively in the reported sequence (54). The cDNA sequence differences were observed at nucleotide positions 264, 392, 393, 852, 854 and 1121, *i.e.* A at 264, C at 392, C at 393, A at 852, A at 854 and C at 1121 in the present study were G, A, T, G, G and T, respectively in the reported sequence (54). Moreover, the cDNA sequence (5'-TTTGGG-3') corresponding to residues 1174-1179 in the present study was TTTTGGGG in the reported sequence (54).

4-3-2 *Comparison of the Amino Acid Sequence of Human Ficolin-1 with Porcine Ficolins, EBP-37, Hucolin and P35*

The overall amino acid sequence of human ficolin-1 showed 74.3% identity to porcine ficolin-α, and 78.1% to porcine ficolin-β by using the Clustal computer alignment program (74) of Lasergene program (DNASTAR, Inc.). Comparison of the mature part revealed that human ficolin-1 has 77.9% amino acid identity with that of porcine ficolin-α and 80.3% with that of porcine ficolin-β. P35 had also a significant homology to porcine ficolins, *i.e.* 74.4% amino acid sequence identity to porcine ficolin-α and 75.1% to ficolin-β, respectively. The overall sequence identity of human ficolin-1 and P35 is 76.7% at the amino acid level (Fig.16). As described in chapter 1, a novel corticosteroid-binding, termed hucolin, was purified from human plasma (28). The partial amino acid sequence obtained from the purified EBP-37 and hucolin all matched the deduced amino acid sequence of P35 (Fig.17), indicating that EBP-37, P35, and hucolin are identical. Phylogenetic comparison suggested that human ficolin-1 is more closely related to human P-35 than porcine ficolins (Fig.18). Thus, by the amino acid sequence comparison, we could not determine whether the human ficolin-1 is a human counterpart

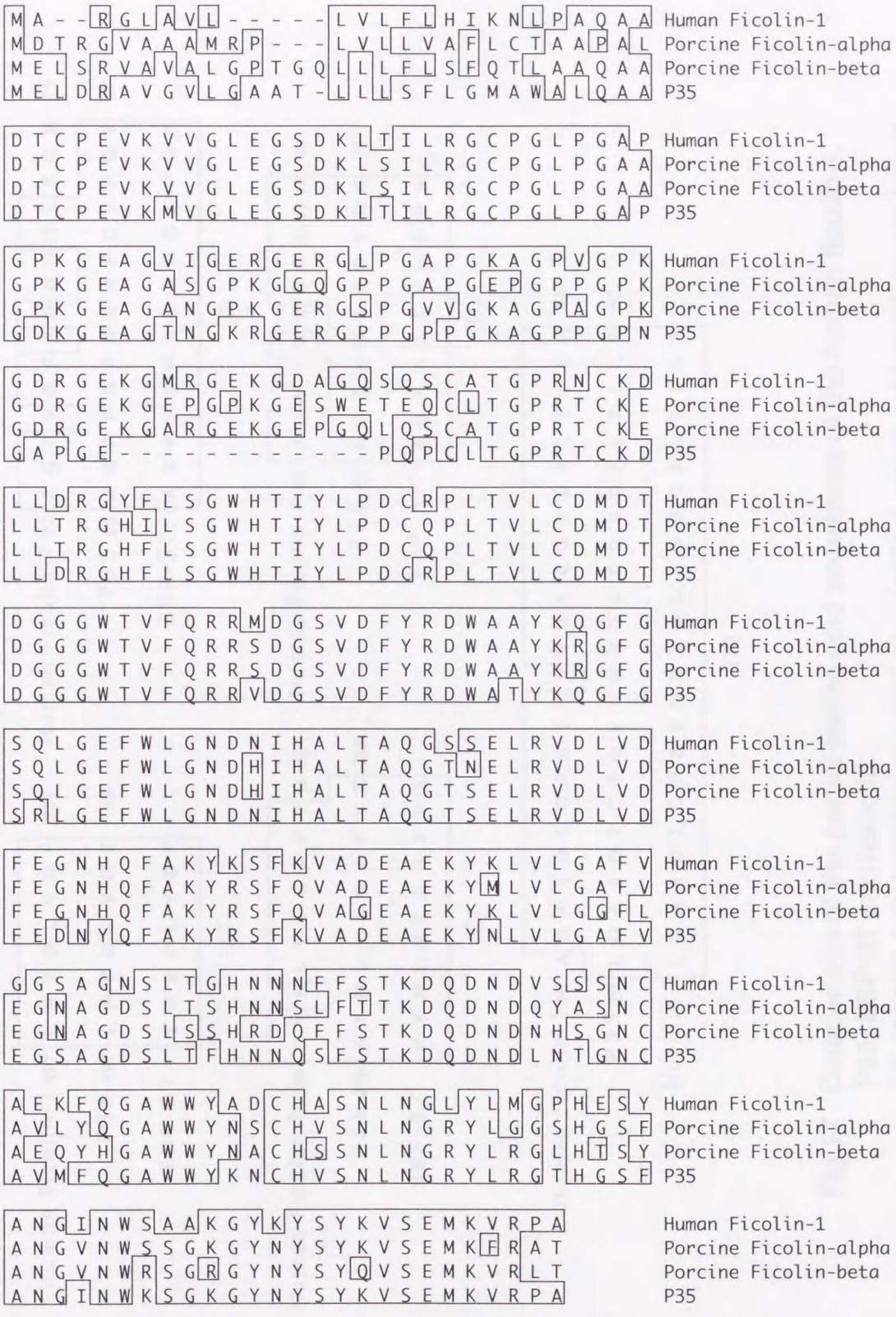


Fig. 16 Comparison of the amino acid sequence of the human ficolin-1 with those of porcine ficolin-α, -β and human P35.
Conserved amino acid residues are boxed.

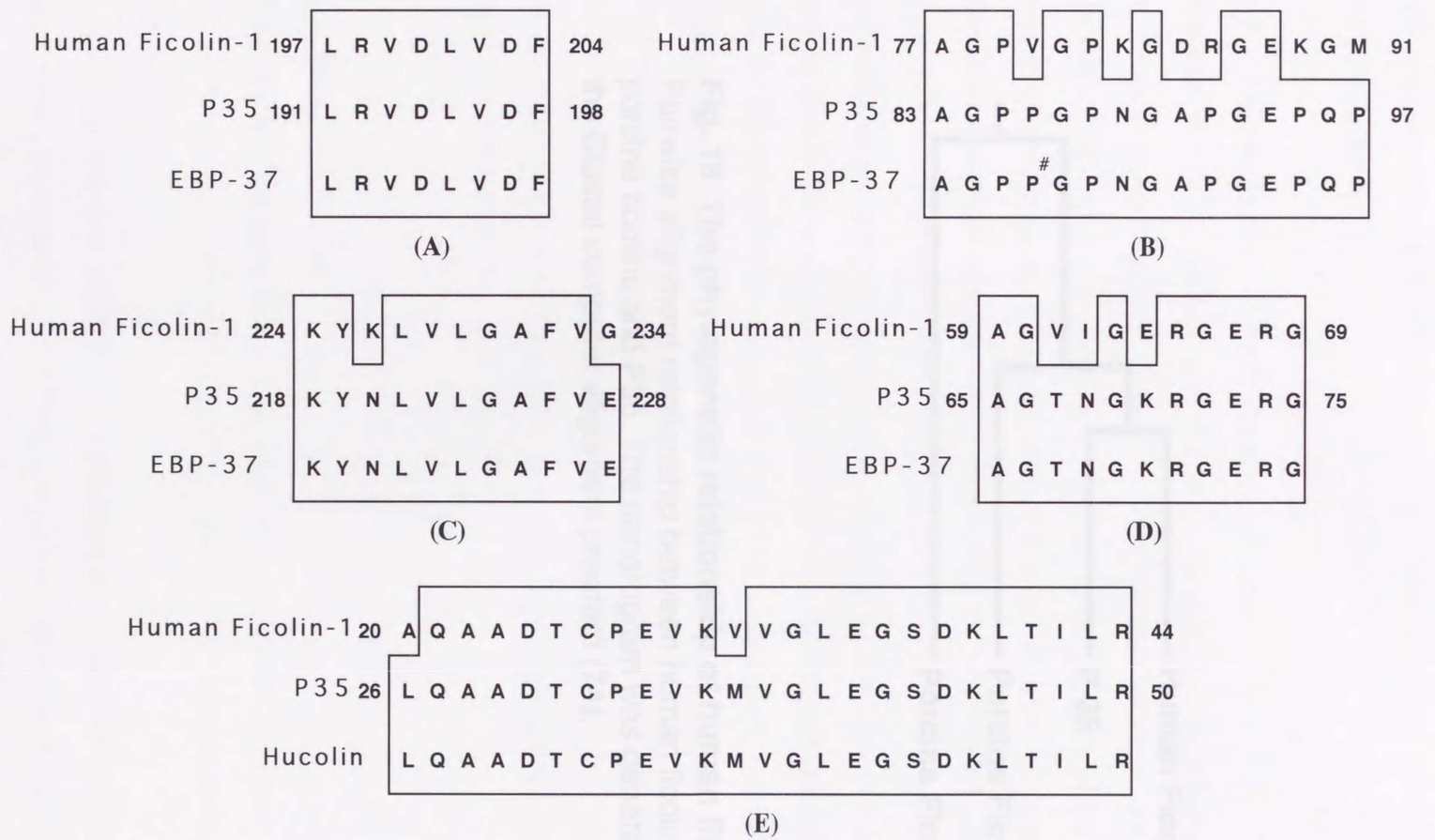


Fig.17 Comparison of the partial amino acid sequences of the human ficolin-1, P35, EBP-37 and hucolin.

P# denotes a hydroxyproline residue. Identical amino acid residues are boxed.

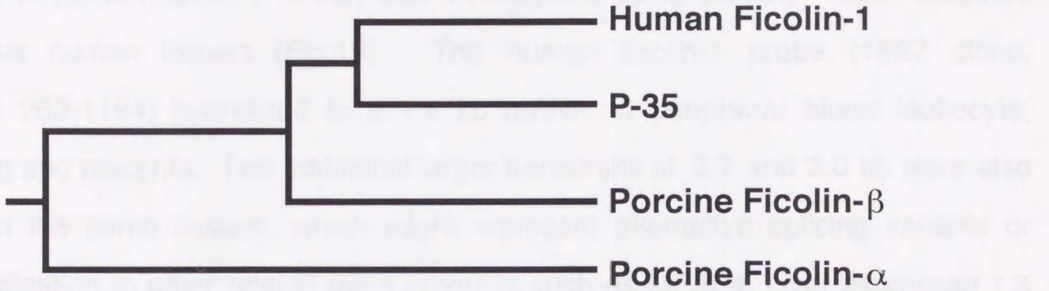


Fig. 18 The phylogenetic relationship of human ficolin-1.
Pairwise alignment relationship between human ficolin-1, porcine ficolins and P35. The dendrogram was generated using the Clustal computer alignment program (74).

of porcine ficolin-α or ficolin-β. Therefore, the human clone was termed ficolin-1. These results suggested that EBP-37/P35/hucolin and human ficolin-1 in this report compose a human ficolin family.

4-3-3 *Expression of Human Ficolin-1 mRNA*

Distribution of human ficolin-1 mRNA was investigated using poly(A)+ RNA obtained from various human tissues (Fig.19). The human ficolin-1 probe (15S7 clone, nucleotides 262-1194) hybridized to a 1.4 kb mRNA in peripheral blood leukocyte, spleen, lung and placenta. Two additional larger transcripts of 3.7 and 3.0 kb were also observed in the same tissues, which might represent alternative splicing variants or cross-hybridization to other related gene products such as MFAP4. Liver expressed 1.3 kb transcript, which is slightly smaller than the major 1.4 kb transcript in peripheral blood leukocyte. Since P35 was mainly expressed in liver, the 1.3 kb message may be a cross-hybridized signal to P35. Expression of P35 in blood leukocyte was not tested (27). The results indicated that the human ficolin-1 is mainly synthesized in peripheral blood leukocyte. Expression of porcine ficolin-α and -β was tested using human MTN blot (24). A 1.4 kb transcript of ficolin-α was found in placenta and lung, and a similar sized transcript of ficolin-β was found in skeletal muscle. Thus, the expression profile of human ficolin-1 is more like ficolin-α than ficolin-β.

4-4 DISCUSSION

In this chapter, I describe the cloning and characterization of human ficolin-1, each of which contains two distinct structural features, *i.e.* a fibrinogen-like domain and a collagen-like domain.

As described in chapter 1, the mammalian molecules containing collagen-like repeats have been reported, including complement C1q, SP-A, MBP, and conglutinin. They are involved in the first line host defense against pathogens.

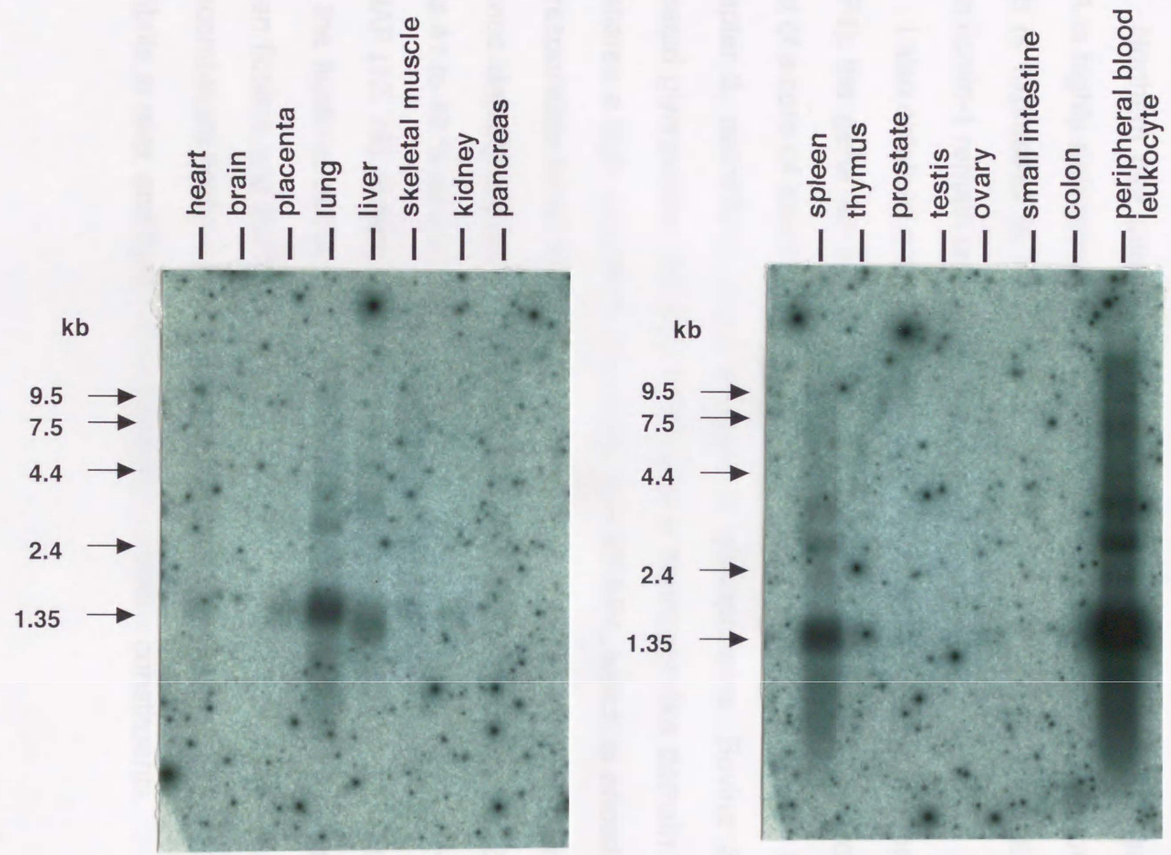


Fig. 19 Northern blot analysis of human ficolin-1.
Human multiple tissue northern (MTN) blot and MTN blot II (Clontech) were probed with the human ficolin-1 cDNA fragment. Each lane contained 2 μg of polyadenylated RNA from the indicated tissues. Size markers are indicated on the left.

It has been reported that fibrinogen-like domains occur in certain proteins such as tenascin and cytotactin (76, 77). Tenascin is well known as an extracellular matrix protein with a dynamic and temporally restricted tissue destribution during embryogenesis (78). However, the precise functions of the fibrinogen-like domain containing proteins have not been fully elucidated.

Northern blot analysis of various human tissues revealed that human ficolin-1 mRNA is highly expressed in peripheral blood leukocytes. The major P35 transcript of 1.3 kb is expressed in liver. The functional differences between EBP-37 (P35) and human ficolin-1 remain unknown.

I also obtained a cDNA that encode a human microfibril-associated glycoprotein (MFAP4); the gene for MFAP4 is deleted in Smith-Magenis syndrome. Elastic fibers consist of a core of elastic protein, elastin, surrounded by microfibrils (65). As described in chapter 3, microfibrils are a complex of glycoproteins. Bovine 36-kDa microfibril-associated glycoprotein (36 kDa MAP) has a fibrinogen-like domain (79). The 36 kDa MAP shares a high sequence homology with MFAP4, which is encoded by a candidate gene responsible for Smith-Magenis syndrome. MFAP-4 revealed 51 to 52 % amino acid sequence idenitity to porcine ficolins in their fibrinogen-like domain (12), and the 36 kDa MAP is 41 to 42 % similar to ficolins in the 127 amino acid sequence available for the 36 kDa MAP (12, 79). In fact, cDNAs for MFAP4 were isolated by screening the cDNA library using the ficolin-α cDNA probe in the present study. Thus, the structural relationship between ficolins and the 36 kDa MAP and MFAP-4, and the binding activity of EBP-37 and recombinant ficolin-α to elastin suggest that ficolins may also associate with elastin-microfibrils *in vivo*, and function as extracellular matrix constituents.

Chapter 5

Interaction of Human Plasma Fibronectin and α-Elastin

5-1 INTRODUCTION

I described about EBP-37 and ficolins as novel elastin-binding proteins. In this chapter, interaction of human plasma fibronectin (pFN) with α-elastin was reported. As described previously, elastin is the core protein of elastic fibers and is a major component of the vessel wall. Various constituents of blood such as immunoglobulins (Igs), lipids, calcium ions (15) and fibrin monomers (16, 17) are known to interact with this protein, and the interactions may play a significant role in atherosclerosis and age-related changes of the human aorta (18, 26).

Fibronectin (FN) is a cell-adhesive protein, and occurs both in plasma and in extracellular matrices. It interacts with various extracellular matrix components such as collagens and glycosaminoglycans, and is implicated in a variety of biological processes (80).

However, whether FN interacts with elastin is not clear. It has been reported that pFN did not bind to κ-elastin-Sepharose or fibrous elastin-Sepharose *in vitro* (81). Conversely, it has been reported that ^{125}I-labeled pFN bound to fibrous elastin *in vitro* and the amount of bound pFN was a direct function of the amount of elastin (16). *In vivo* studies have shown the topographic association of FN with elastic fibers in the arterial wall (82) and the detection of FN at the periphery of elastic fibrils (83). I report here that pFN can bind to α-elastin by hydrophobic interaction under certain conditions *in vitro*.

5-2 MATERIALS AND METHODS

MATERIALS

Gelatin-Sepharose 4B was purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. *n*-Propylamine, *n*-butylamine, *n*-hexylamine, and CNBr were from Wako Pure Chemicals, Osaka. Plasma fibronectin (pFN) was purified from normal human plasma by gelatin affinity chromatography. Rabbit anti-bovine α-elastin antibody was kindly provided by Dr. Toshiro Ooyama, Toho University School of Medicine. All other chemicals were of the purest grade commercially available.

Preparations of α-Elastin and α-Elastin-Sepharose 4B

α-Elastin (25) and α-elastin-Sepharose 4B (21) were prepared as described previously.

α-Elastin-Sepharose Affinity Chromatography of Human Plasma

The chromatography was performed either at 25°C or 4°C. Human plasma (120ml) mixed with 0.6ml of 0.2M phenylmethanesulfonyl fluoride in ethanol was passed through a Sepharose 4B column (1.1x5.3cm) and then applied to an α-elastin-Sepharose column (1.1x5.3cm) equilibrated with 0.1M NaCl-0.05M Tris-HCl, pH7.4 (Tris-buffered saline(TBS)) at 25°C or 4°C . After being washed with TBS, the column was eluted with 4M urea-0.05M Tris-HCl, pH7.4.

α-Elastin-Sepharose Affinity Chromatography of pFN

pFN (1.4mg) was applied to an α-elastin-Sepharose column (0.8x4.7cm) eqilibrated with TBS. After being washed with TBS, the column was successively eluted with 1M NaCl-0.05M Tris-HCl (pH7.4 at 25°C or 4°C), 1M KBr-0.05M sodium acetate buffer (pH5.3 at 25°C or 4°C) and 8M urea-0.05M Tris-HCl (pH7.4 at 25°C or 4°C) in a stepwise manner.

Protein Determination

Protein concentrations of pFN were estimated spectrophotometrically at 280nm based on an absorption coefficient (12.8) (84) and that of proteins other than fibronectin by using the Bio-Rad protein assay kit (Bio-Rad Laboratories, Richmond, CA), with bovine serum albumin as a standard.

Electrophoresis

SDS-PAGE was performed according to the method of Laemmli (32) in 7.5% acrylamide gel after reduction with 1% 2-mercaptoethanol. Native PAGE was performed in 5% acrylamide gel (85). The gels were stained with CBB R-250 (Merck) or a silver stain kit (Wako).

Electrophoretic Transfer of Proteins to PVDF Membranes

After SDS-PAGE or native PAGE, proteins were transferred to PVDF membranes as described previously.

Immunological Detection of pFN

The membrane was washed with phosphate-buffered saline(PBS) containing 0.05% Tween-20(PBST) for 5min three times, and then soaked in PBS containing 3%(w/v)bovine serum albumin(pH7.3) overnight at 4°C. It was washed with PBST three times, and immunostained with an anti-pFN antibody($F(ab')_2$ fragment, Cappel), diluted 1/1000 in 1% bovine serum albumin in PBS, pH7.4. Biotin-SP-Affinipure $F(ab')_2$ fragment rabbit anti-goat IgG(Jackson) diluted 1/2000 in the same solution was used as a second antibody. Streptavidin-horseradish peroxidase conjugate(GIBCO BRL) diluted 1/1000 in the same solution and 0.05% 3,3'-diaminobenzidine, 0.045% H_2O_2, and 0.84mM $CoCl_2$ in 10mM Tris-HCl, pH7.5 were used to detect the second antibody.

Assay for Binding of α-Elastin to pFN on PVDF Membrane

Binding assay was performed essentially according to the method of Heremans *et al.* (86). pFN (5μg or 25μg) was subjected to native PAGE. After electrophoresis, pFN was electrotransferred onto PVDF membranes. Then, the membranes were inactivated with 1%(w/v)casein in phosphate-buffered 0.6M NaCl, pH7.4. The membranes were incubated with 4%(w/v)α-elastin in 0.1M NaCl-0.05M Tris-HCl(pH7.4) for 12h at 4°C on a shaker, and then immunostained with an anti-α-elastin antibody diluted 1/500 in 0.5%(w/v)casein in PBS, pH7.4. Biotin-labeled goat anti-IgG(Jackson, U.S.A.) diluted 1/1500 in the same solution was used as a second antibody and streptavidin-horseradish peroxidase conjugate diluted 1/1000 in the same solution and 0.05% 3,3'-diaminobenzidine, 0.045% H_2O_2 and 0.84mM $CoCl_2$ in 10mM Tris-HCl, pH7.5 were used to detect the second antibody.

Preparation of Alkyl-Sepharose 4B

Alkyl-Sepharoses with hydrophobic chains of various lengths were prepared essentially according to the method of Shaltiel (87) by coupling *n*-alkylamines to CNBr-activated Sepharose 4B.

Alkyl-Sepharose Hydrophobic Chromatography of pFN

pFN (0.9-1.4mg) was applied to each alkyl-Sepharose column (0.8x4.7cm) equilibrated with TBS. After being washed with TBS, the column was successively eluted with 1M NaCl-0.05M Tris-HCl (pH7.4 at 25°C or 4°C), 1M KBr-0.05M sodium acetate buffer (pH5.3 at 25°C or 4°C) and 8M urea-0.05M Tris-HCl (pH7.4 at 25°C or4°C), in a stepwise manner.

5-3 RESULTS

5-3-1 *α-Elastin-Sepharose Affinity Chromatography of Human Plasma*

Elution profiles of α-elastin-Sepharose affinity chromatography at 4°C and 25°C are shown in Fig.20. The amount of bound proteins at 4°C was 15-20 times more than that at 25°C, *i.e.* 25-30mg of protein at 4°C and 1.5-2.0mg of protein at 25°C from 100ml of plasma.

5-3-2 *SDS-PAGE and Immunoblot Analysis of α-Elastin-Sepharose Binding Proteins*

SDS-7.5% polyacrylamide gel electrophoresis under reducing conditions indicated that bound proteins both at 4°C (Fig.21 A(a)) and at 25°C (Fig.21 B(a)) contained pFN and Igs (IgG, IgM *etc.*). The presence of pFN was confirmed by electrophoretic transfer and immunological detection with anti-FN antibody (Fig.21 A(b) and 21 B(b)). Apparently the amount of the bound pFN at 4°C was more than that at 25°C.

5-3-3 *Interaction of Purified pFN with α-Elastin-Sepharose*

In order to examine whether pFN interacts with α-elastin-Sepharose directly, pFN was isolated and then was applied to the α-elastin-Sepharose column. Elution patterns are shown in Fig.22. At 4°C, most of the pFN was bound to the column. Total recovery was about 70% in terms of absorbance at 280nm. At 25°C, most of the pFN passed through the column. When pFN was applied to a plain Sepharose 4B column at 4°C and 25°C, 95-96% of it passed through the column in terms of absorbance at 280nm. The results indicate that pFN can bind directly to α-elastin-Sepharose at 4°C, whereas the binding is weaker at 25°C.

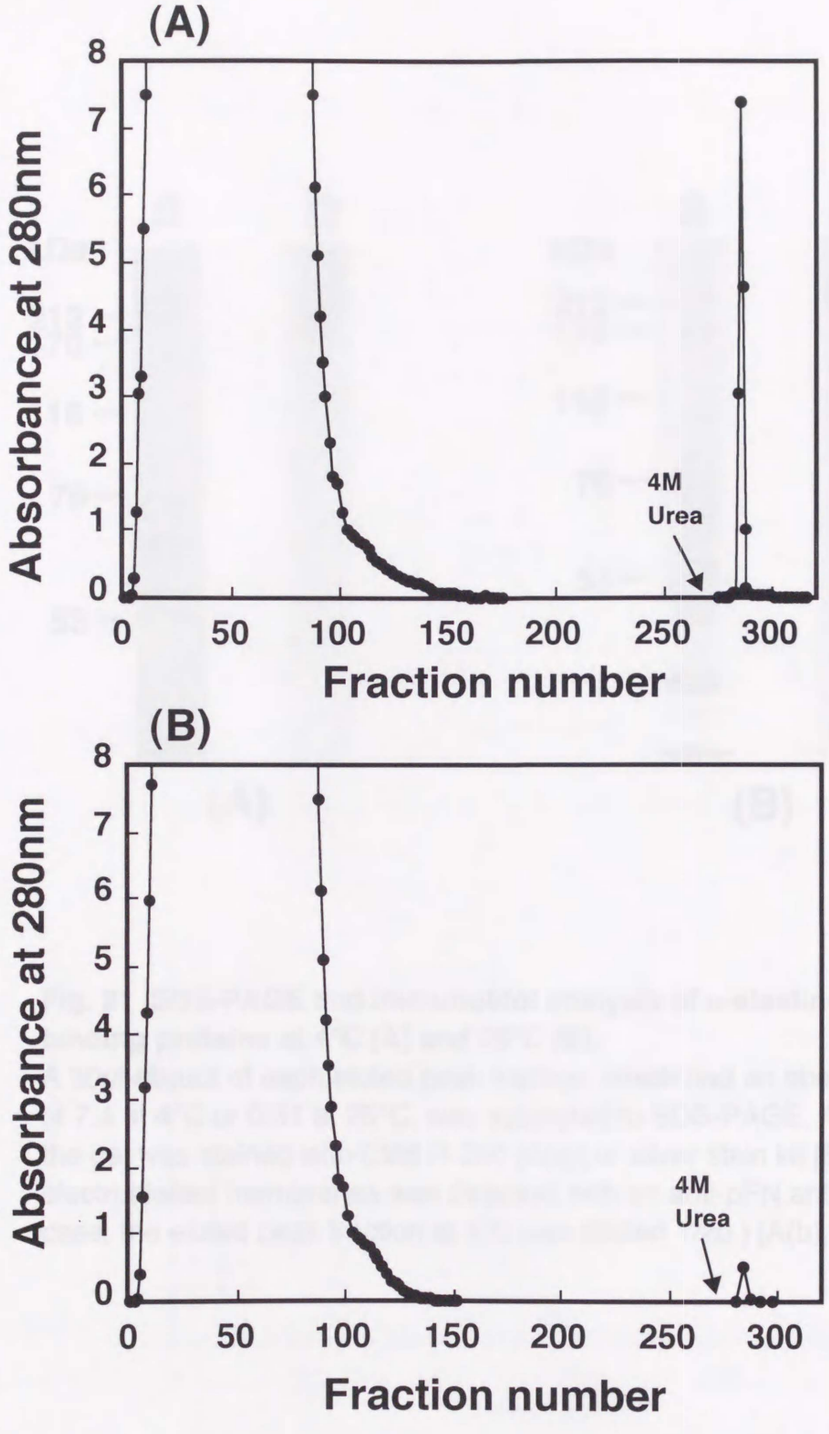


Fig.20 α-Elastin-Sepharose affinity chromatography of human plasma at 4°C (A) or at 25°C (B).

Human plasma (120ml) was applied to a 5ml column of untreated Sepharose and then to an α-elastin-Sepharose column, which was eluted with 4M urea (indicated by the arrows). Fractions of 1.4ml each were collected. Details of the procedure are described in the text.

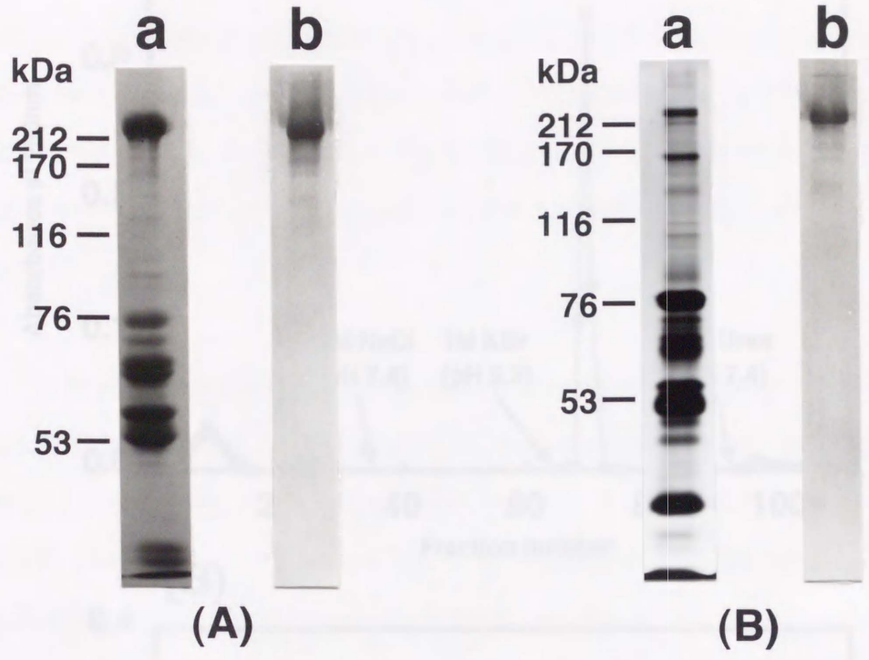


Fig. 21 SDS-PAGE and immunoblot analysis of α-elastin-Sepharose binding proteins at 4°C (A) and 25°C (B).
A 30μl aliquot of each eluted peak fraction, which had an absorbance value of 7.4 at 4°C or 0.51 at 25°C, was subjected to SDS-PAGE. After SDS-PAGE, the gel was stained with CBB R-250 [A(a)] or silver stain kit [B(a)]. pFN on electroblotted membranes was detected with an anti-pFN antibody (In this case, the eluted peak fraction at 4°C was diluted 1/20.) [A(b),B(b)].

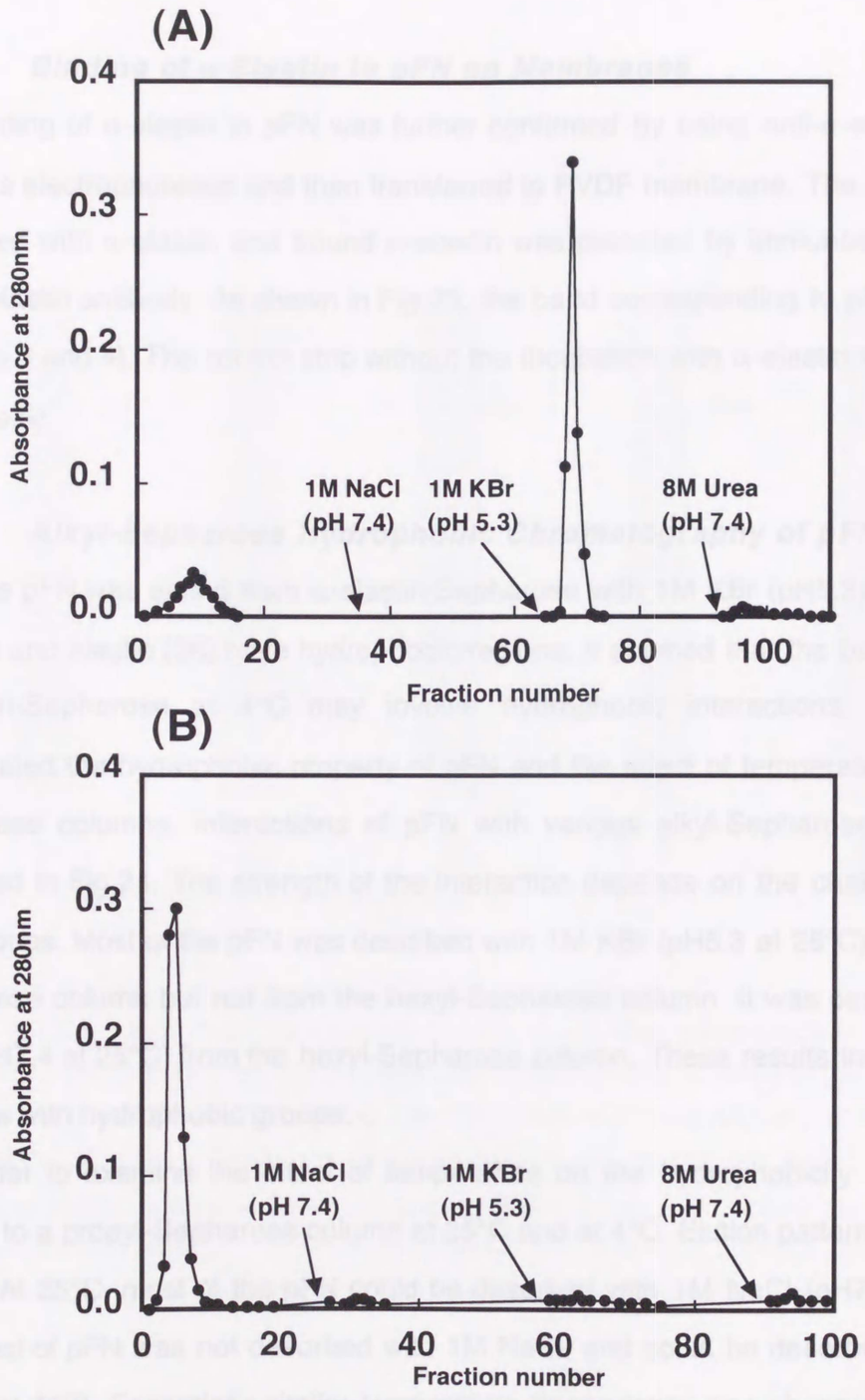


Fig. 22 Elution pattern of pFN from an α-elastin-Sepharose column at 4°C (A) and 25°C (B).

pFN (1.4mg) was applied to an α-elastin-Sepharose column (2ml column). After washing of the column, pFN was eluted as described in the text. Fractions of 1.5ml each were collected.

5-3-4 *Binding of α-Elastin to pFN on Membranes*

The binding of α-elastin to pFN was further confirmed by using anti-α-elastin antibody. pFN was electrophoresed and then transferred to PVDF membrane. The membrane was incubated with α-elastin and bound α-elastin was detected by immunostaining with an anti-α-elastin antibody. As shown in Fig.23, the band corresponding to pFN was stained (lane No.3 and 4). The control strip without the incubation with α-elastin was not stained (lane No.5).

5-3-5 *Alkyl-Sepharose Hydrophobic Chromatography of pFN*

Because pFN was eluted from α-elastin-Sepharose with 1M KBr (pH5.3), and both pFN (88, 89) and elastin (26) have hydrophobic regions, it seemed that the binding of pFN to α-elastin-Sepharose at 4°C may involve hydrophobic interactions. Therefore, we investigated the hydrophobic property of pFN and the effect of temperature using alkyl-Sepharose columns. Interactions of pFN with various alkyl-Sepharoses at 25°C are illustrated in Fig.24. The strength of the interaction depends on the chain length of the alkyl groups. Most of the pFN was desorbed with 1M KBr (pH5.3 at 25°C) from the butyl-Sepharose column but not from the hexyl-Sepharose column. It was desorbed with 8M urea (pH7.4 at 25°C) from the hexyl-Sepharose column. These results indicate that pFN interacts with hydrophobic groups.

In order to examine the effect of temperature on the hydrophobicity of pFN, it was applied to a propyl-Sepharose column at 25°C and at 4°C. Elution patterns are shown in Fig.25. At 25°C, most of the pFN could be desorbed with 1M NaCl (pH7.4 at 25°C). At 4°C, most of pFN was not desorbed with 1M NaCl, and could be desorbed with 1M KBr (pH5.3 at 4°C). Essentially similar temperature dependency was observed in butyl- and hexyl-Sepharose chromatography (data not shown).

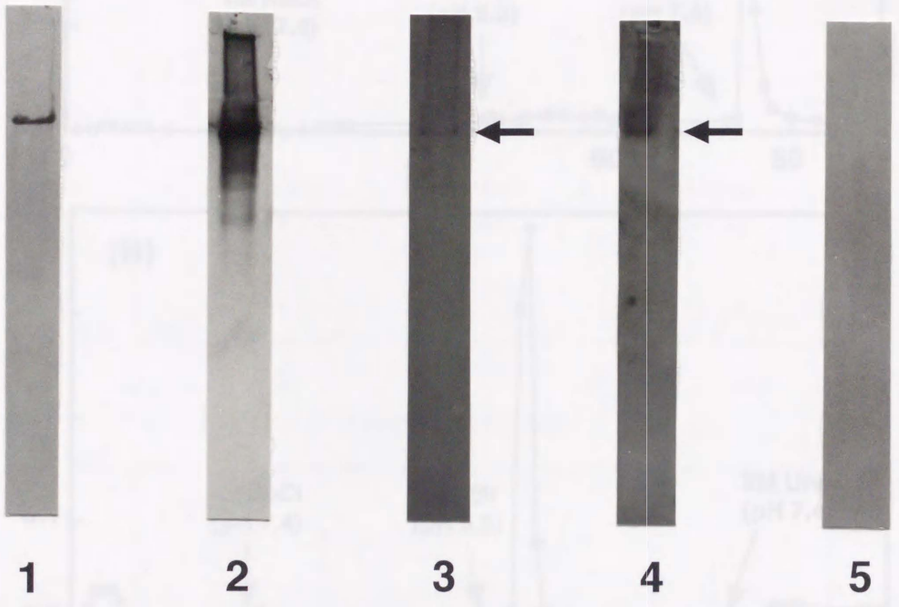


Fig.23 Binding of α-elastin to pFN on membranes.
pFN [5μg (lanes No.1,2,3,5) or 25μg (lane No.4)] was electroblotted onto PVDF membranes after native electrophoresis in a 5% acrylamide gel. Then, membrane strips (lane No.3 and 4) were incubated with α-elastin solution. Bound α-elastin was detected by immunostaining with an anti-α-elastin antibody. Other strips were stained with amido black (lane No.1) or with an anti pFN antibody (lane No.2). Lane No.5 shows a control stained with an anti-α-elastin antibody without incubation with α-elastin.

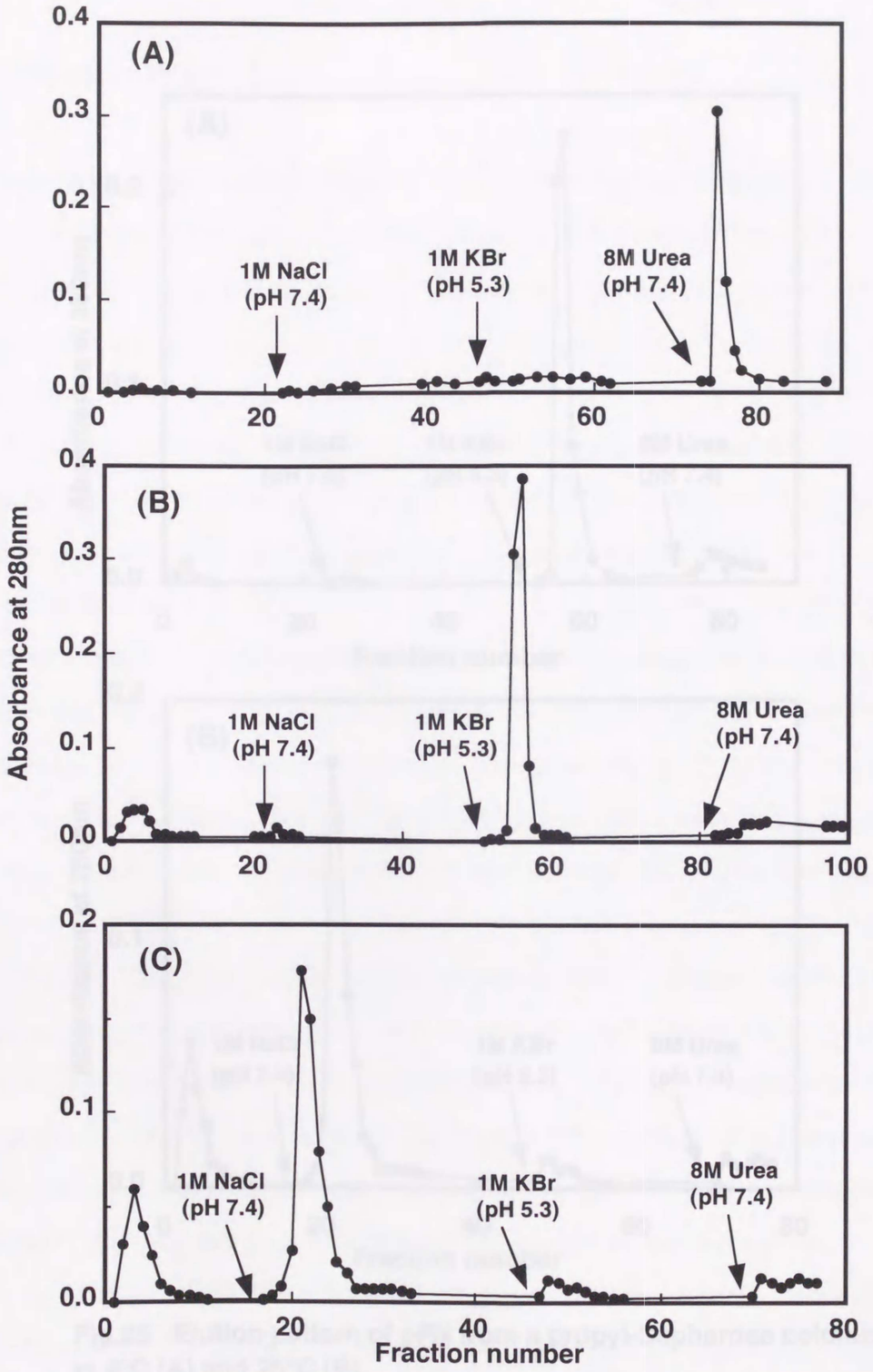


Fig.24 Elution patterns of pFN from alkyl-Sepharose columns at 25°C.
pFN (1.4mg [hexyl-Sepharose(A)], 1.2mg [butyl-Sepharose(B)], 0.9mg [propyl-Sepharose(C)]) was applied to each column (2ml column). After washing of the column, pFN was eluted as described in the text. Fractions of 1.5ml each were collected.

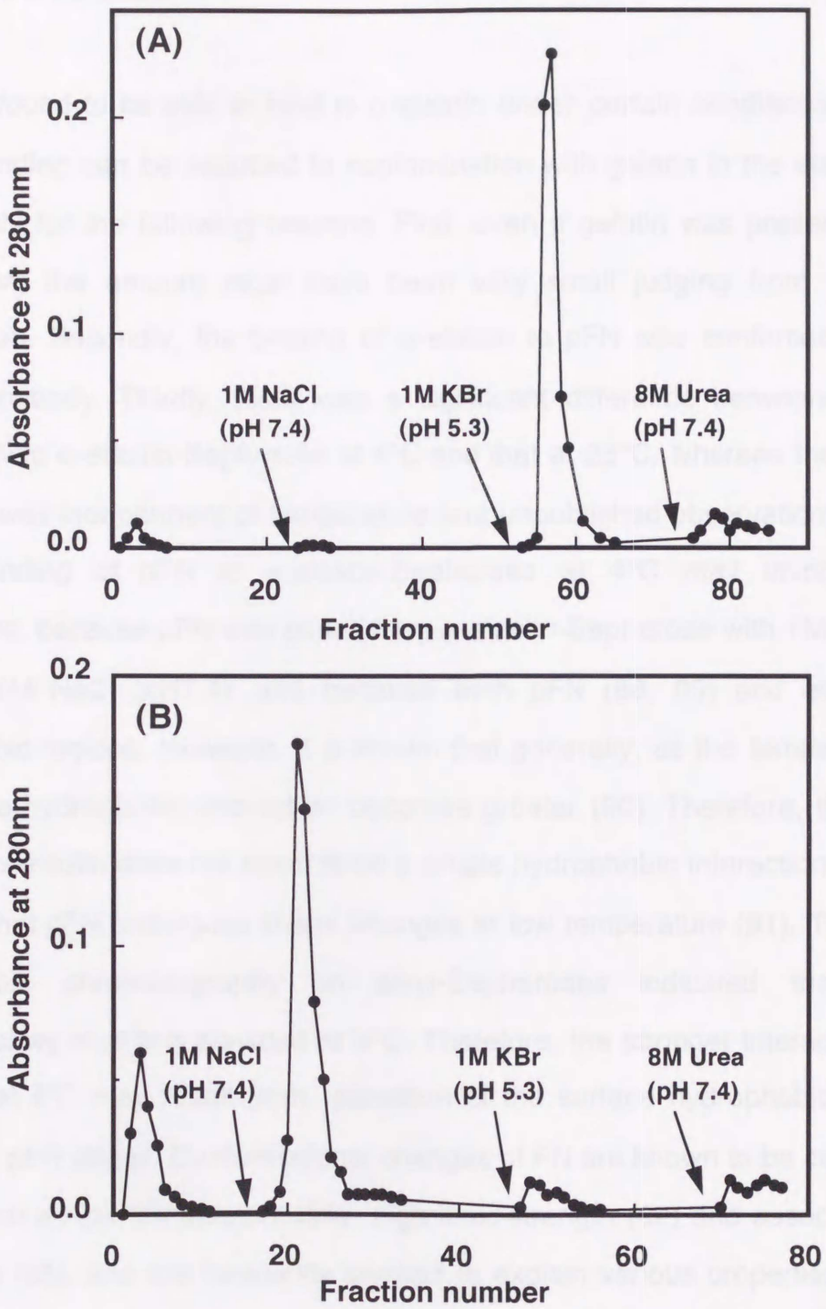


Fig.25 Elution pattern of pFN from a propyl-Sepharose column at 4°C (A) and 25°C (B).

pFN (0.9mg) was applied to a propyl-Sepharose column (2ml column). After washing of the column, pFN was eluted as described in the text. Fractions of 1.5ml each were collected.

5-4 DISCUSSION

pFN was found to be able to bind to α-elastin under certain conditions. The possibility that the binding can be ascribed to contamination with gelatin in the elastin preparation is not likely for the following reasons. First, even if gelatin was present in the elastin preparation, the amount must have been very small judging from the amino acid composition. Secondly, the binding of α-elastin to pFN was confirmed with α-elastin-specific antibody. Thirdly, there was a significant difference between the amount of bound pFN to α-elastin-Sepharose at 4°C and that at 25°C, whereas the binding of pFN to gelatin was independent of temperature (our unpublished observation).

The binding of pFN to α-elastin-Sepharose at 4°C may involve hydrophobic interactions, because pFN was eluted from α-elastin-Sepharose with 1M KBr (pH5.3) but not with 1M NaCl (pH7.4), and because both pFN (88, 89) and elastin (26) have hydrophobic regions. However, it is known that generally, as the temperature becomes higher, the hydrophobic interaction becomes greater (90). Therefore, the interaction of pFN with α-elastin does not seem to be a simple hydrophobic interaction. Mortillaro *et.al.* reported that pFN undergoes shape changes at low temperature (91). The results of the hydrophobic chromatography on alkyl-Sepharoses indicated that the surface hydrophobicity of pFN is elevated at 4°C. Therefore, the stronger interaction of pFN with α-elastin at 4°C may result from elevation of the surface hydrophobicity caused by a change of pFN shape. Conformational changes of FN are known to be caused by various factors such as low temperature (91), high ionic strength (92) and association with other molecules (93), and are frequently invoked to explain various properties of pFN. Direct analyses of the contributions of such changes will be essential to understand the interaction of FN with various macromolecules involved in fibrillogenesis (93).

The interaction of FN with elastin has been studied immunohistochemically, and topographic association of FN with the external and internal elastic lamina of large vessel walls (82) and detection of FN at the periphery of elastic fibrils and in amorphous,

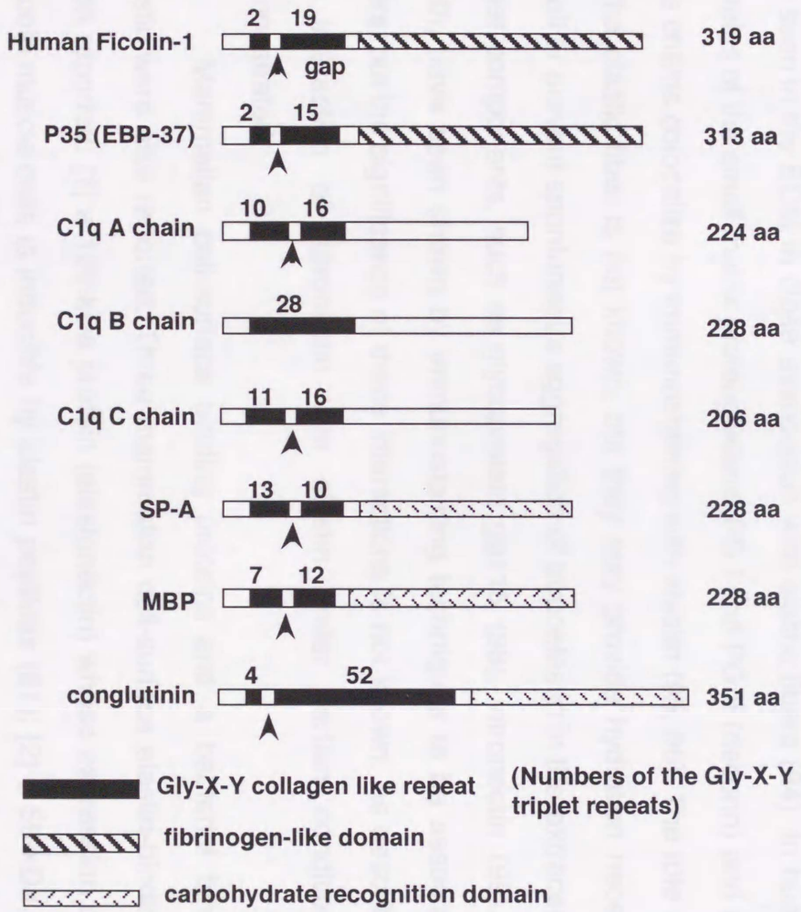
non-fibrillar regions of skin (83) were described. These findings suggests that FN is associated with elastic fibers *in vivo* under certain conditions.

GENERAL DISCUSSION

Although elastic fibers are found in connective tissue of most organs, they are particularly abundant in tissues that are subjected to repetitive deformation. The blood vessel wall is an elastic tissue, and the elastic protein elastin provides for energy-dependent elastic recoil with pulse pressure. Elastic fibers are denatured by binding of various constituents of blood to elastin.

In the present study, I found a novel elastin-binding protein (EBP-37) in human plasma by using α-elastin-Sepharose affinity chromatography. EBP-37 is highly similar but not identical to porcine ficolins. Recently, a novel human serum lectin, termed P35, was molecularly cloned (27). Comparison of the amino acid sequence of EBP-37 with that of P35 indicated that these proteins are identical. In addition, a novel corticosteroid-binding protein, termed hucolin, was purified from human plasma, and its N-terminal amino acid sequence was determined recently (28). Human hucolin also appears to be identical to EBP-37 and P35.

In this study, a cDNA encoding a human counterpart of porcine ficolins that is composed of 319 amino acids and is different from EBP-37 was cloned and named human ficolin-1. Human ficolin-1 cDNA is closely related to, but different from EBP-37/P35/hucolin. Northern blotting of various human tissues revealed that human ficolin-1 mRNA is highly expressed in peripheral blood leukocytes. The deduced amino acid sequence of human ficolin-1 has the features of a secreted protein, starting with a hydrophobic signal sequence. The function and precence of the human ficolin-1 gene product in the circulation and other body fluids remain unknown. These results suggested that EBP-37/P35/hucolin and human ficolin-1 in this study compose a human ficolin family. The functional differences betweem EBP-37 (P35) and human ficolin-1 remain unknown. Schematic illustrations of human ficolin-1, P35, and certain other collagen-like domain containing proteins were shown (Fig.26).



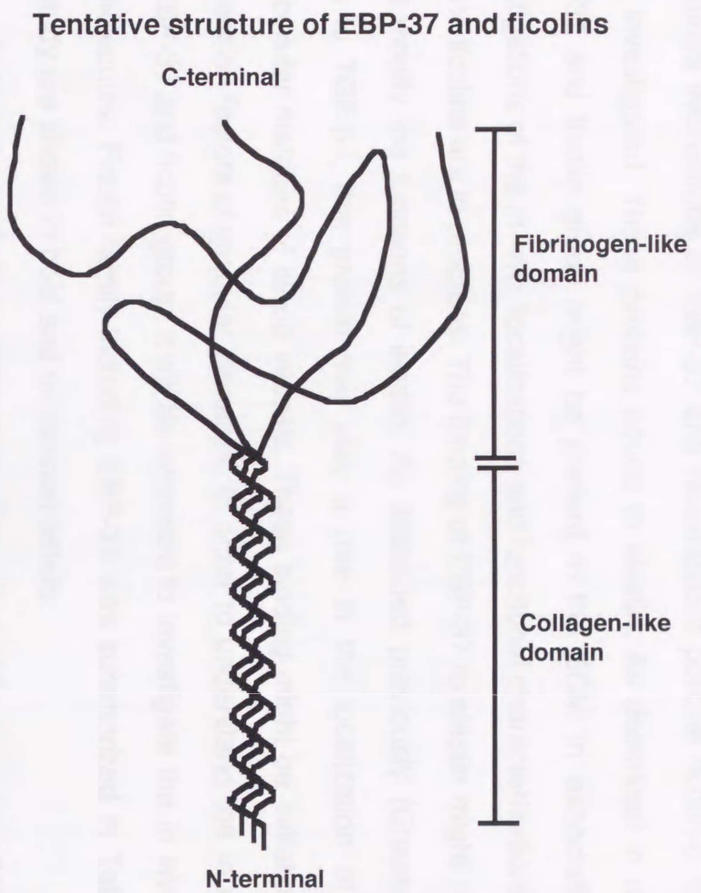


Fig.26 Schematic illustration of human ficolin-1, P35, and certain other collagen-like domain containing proteins. Human ficolin-1, P35, complement C1q, human pulmonary surfactant apoprotein (SP-A), human mannan-binding protein (MBP), and bovine conglutinin are schematically aligned. Tentative structure of EBP-37 and ficolins.

The direct interactions of EBP-37 and recombinant porcine ficolin-α to elastin *in vitro* were investigated. These proteins bound to elastin. As described in chapter 3 and 4, EBP-37 and ficolin group might be present in the ECM in association with elastin. Investigations of the *in vivo* localizations and functional characterizations of EBP-37 and porcine ficolins are in progress. The binding of EBP-37 to elastin might occur *in vivo* and might modify the functions of elastin. As described previously (Chapter 1), if EBP-37 binds to TGF-β1, this protein may play a role in the localization of TGF-β1 in the extracellular matrices of blood vessels. These binding might be initiation factors and/or progression factors of vascular diseases. In order to understand the *in vivo* functions of the EBP-37 and ficolin group, it will be important to investigate the *in vivo* localization of the molecules. Ficolin family including EBP-37 was summarized in Table 2. Results of this study are shown in bold and underlined letters.

Associations of other matrix molecules with elastin were reported. Proteoglycans are seen in the ECM in close association with elastic fibers (94). In human dermis, core proteins of the small matrix proteoglycans, PG I and PG II (decorin) and dermatan sulfate side chains colocalize by immunostaining with elastin (95, 96). The role of proteoglycans in the elastic fiber is not known, but they may provide hydration necessary for elastic recoil or prevent spontaneous aggregation of tropoelastin in the extracellular space (97). Other components, such as glycoprotein gp115 (98), vitronectin (99), and amyloid P (100), have been shown by immunostaining techniques to be associared with elastic fibers, but the significance of these interactions is not known. As described in chapter 5, the interaction of fibronectin with elastin under certain conditios *in vitro* was demonstrated.

Mammalian cell-surface binding proteins and a bacterial binding protein for elastin were also reported. Three mammalian cell-surface elastin-binding proteins have been reported: [1] a 120-kDa protein (elastonectin) whose expression on the surface of smooth muscle cells is inducible by elastin peptides (81); [2] a 59-kDa protein found on tumor cells that is coupled to protein kinase C (101); [3] a 67-kDa peripheral membrane

Table 2 Summary of Ficolin Family				
	Protein	cDNA	Occurrence	Biological Activity
Human	**EBP-37** P35 Hucolin		**Plasma** (Serum)	**Elastin-Binding** Lectin Opsonin Corticosteroid-Binding
		P35	Liver	
		Ficolin-1	**Peripheral BloodLeukocyte**	**?**
Pig	Ficolin-α	Ficolin-α	Uterus	TGF-β1-Binding (?) **Elastin-Binding**
		Ficolin-β	Uterus	?
	Ficolin-α (?)		**Plasma**	**Elastin-Binding**

Bold and underlined: Results of this study

protein found on most cell types that bind elastin (59). The 67-kDa elastin-binding protein colocalizes with tropoelastin in intracellular vesicles leading to the hypothesis that the receptor serves as a molecular chaperone. As described in chapter 3, this protein may be identical to the catalytically inactive variant of β-galactosidase produced by alternative splicing (61). It was elucidated that the domain which corresponds to a frameshift-generated sequence unique to the alternatively spliced variant is responsible for its elastin-binding properties. Elastin contains no Arg-Gly-Asp (RGD) sequences, and there is no evidence that elastin interacts with integrins. The integrins bind to ECM proteins at specialized cell attachment sites that often have the tripeptide sequence RGD as the target sequence for the integrin binding.

Many bacteria, including *Staphylococcus aureus*, are known to infect and colonize elastin-rich organs like lung, skin, and blood vessels. The specific association of *Staphylococcus aureus* with elastin was reported (102). A prominent bacterial-binding domain has been mapped to a 30-kDa region at the amino end of elastin molecule (102), obviously different from the mammalian binding protein binding region.

As described previously, various constituents of blood are known to interact with elastin and the interactions have been immunohistochemically demonstrated. It will be important to study the *in vivo* significance of the interactions of EBP-37 and ficolins with elastin.

As described in chapter 2, a large amount of porcine plasma ficolins was identified in the α-elastin-Sepharose-binding fraction of porcine plasma. Ficolin-like proteins could not be detected in α-elastin-binding fractions of sheep, rat, bovine, and goat plasmas by immunoblot analysis. The reasons for the results are unknown. The biological significance of porcine plasma ficolins remains to solved in the future.

ACKNOWLEDGEMENTS

First of all, I sincerely thank Professor Daisaburo Fujimoto, Tokyo University of Agriculture and Technology, my supervisor for his guidance and promotion with all this thesis, and thank professor Yoshiyuki Sakano, Tokyo University of Agriculture and Technology, and Professor Hideyuki Tanaka, Utsunomiya University, for some guidances and correction of this thesis.

I thank Dr. Kohei Miyazono, Dr. Hidenori Ichijo, and Dr. Kohsuke Takeda, the Cancer Institute, for their kind guidance and encouragement during the course of cDNA cloning.

I would like to express my heartfelt thanks to Professor Toshiro Ooyama, Toho University, for useful suggestions and encouragement.

I am thankful to Dr. Akira Oomori, Mitsubishi Kasei Institute of Life sciences, for his invaluable guidance and amino acid sequencing.

I am also thankful to all members of the Laboratory of Biological Chemistry for their help and cooperation in this study.

Finally, I would like to thank my family for their support and encouragement.

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