Microfibril-associated Protein 4 Is Present in Lung Washings and Binds to the Collagen Region of Lung Surfactant Protein D*

Mette Lausen‡, Nicholas Lynch§, Anders Schlosser‡, Ida Tornøe‡, Susanne Gjørup Sækmose‡, Børge Teisner‡, Antony C. Willis‡, Erika Crouch¶, Wilhelm Schwaebles, and Uffe Holmskov**

From the ‡Department of Immunology and Microbiology, Institute of Medical Biology, University of Southern Denmark, Odense University, DK-5000 Odense, Denmark, the §Department of Microbiology and Immunology, University of Leicester, P. O. Box 138, University Road, Leicester LE1 9HN, United Kingdom, the ¶Medical Research Council Immunochemistry Unit, Department of Biochemistry, University of Oxford, South Parks Road, Oxford OX1 3QU, United Kingdom, and the ‡Department of Pathology, Washington University, St. Louis, Missouri 63110

We have purified a glycoprotein from bovine lung washings using affinity chromatography on a maltose-affinity column. On SDS-polyacrylamide gel electrophoresis the protein showed a molecular mass of 36 kDa in the reduced state and 66 kDa in the unreduced state. On gel permeation chromatography the apparent molecular mass was 250 kDa. N-terminal sequencing showed homology to the human matrix protein microfibril-associated protein 4 (hMFAP4), and the glycoprotein was designated bovine MFAP4 (bMFAP4). Lung surfactant protein D (SP-D) was also purified from lung washings, and calcium-dependent binding was demonstrated between hMFAP4 and SP-D. hMFAP4 was cloned, and recombinant hMFAP4 showed the same binding pattern to SP-D as bMFAP4. No binding was seen to recombinant SP-D composed of the neck region and carbohydrate recognition domain of SP-D, indicating that the interaction between MFAP4 and SP-D is mediated via the collagen region of SP-D. MFAP4 also showed calcium-dependent binding to mannan, which was partially inhibited by maltose. Our findings indicate that MFAP4 has two binding specificities, one for collagen and one for carbohydrate, and we suggest that MFAP4 may fix the collectins in the extracellular compartment during inflammation.

Lung surfactant protein D (SP-D) belongs to a group of proteins known as collectins (1, 2). These are C-type lectins containing collagen-like regions attached to carbohydrate recognition domains (CRDs) (3). SP-D is together with lung surfactant protein A (SP-A) mainly found in the surfactant lining the alveolar epithelium, but both molecules are also produced by cells lining the gastrointestinal tract (4, 5). Three serum collectins are known, mannan-binding lectin, conglutinin, and collectin-43, which are all produced by the liver (6, 7). The collectins are structurally related to C1q (8), a subcomponent of C1, and to the ficolins (9). The ficolins differ from the collectins in having a C-terminal fibrinogen-like domain attached to a collagen region. The fibrinogen-like domain of the ficolins is responsible for the carbohydrate binding activity of the ficolins and contains one potential calcium binding site (10–12).

The C-terminal CRDs of the collectins bind to carbohydrate ligands on the surface of pathogens, whereas the collagen region interacts with cell surface receptors to trigger phagocytosis or oxidative killing. SP-A and SP-D also act as chemotactic agents for phagocytes, SP-D being far more potent than SP-A (13–15), and both molecules bind directly to alveolar macrophages in the absence of microbial ligands, thereby mediating the generation of oxygen radicals (14, 16). Different receptors have been described for both SP-A and SP-D (17–20), but it is still not clear which receptors are responsible for the various effector mechanisms elicited by SP-A and SP-D. The main role of SP-D was long thought to be in innate defense against microorganisms, but recent results with SP-D knock-out mice have shown that SP-D is more involved in surfactant homeostasis than previously predicted (21, 22).

Here we describe the purification and characterization of a molecule from bovine lung washings that binds calciumdependently to the collagen regions of SP-D. This molecule also shows calcium-dependent binding to mannan, which is partially inhibited by maltose. The molecule was identified as the bovine homologue to human microfibril-associated protein 4 (hMFAP4) and was therefore designated bovine microfibril-associated protein 4 (bMFAP4). hMFAP4 contains a fibrinogen-like region with high homology to the fibrinogen-like region of the ficolins (23). The ligand motif Arg-Gly-Asp for cell surface integrins is found in the N-terminal region of MFAP4. MFAP4 is the second member of the fibrinogen domain superfamily that shows lectin-like activity, and the possible roles of this activity together with the collagen binding activity and the potential integrin binding activity are discussed.

EXPERIMENTAL PROCEDURES

Buffers and Reagents—Buffers and reagents used were Tris-buffered saline (TBS): 140 mM NaCl, 10 mM Tris-HCl, 0.02% (w/v) NaN3, pH 7.4; TBS/Tw: TBS containing 0.05% (w/v) Tween 20 (polyoxyethylene sorbitol monolaurate, Merck-Schuchardt, Germany); TBS/E: TBS containing 0.05% Emulphogene BC-720 (polyoxyethylene triglyceryl ether, Sigma); phosphate-buffered saline: 137 mM NaCl, 3 mM KCl, 8 mM Na2HPO4, 1.5 mM KH2PO4, pH 7.4; coating buffer: 60 mM Na2CO3, 3.5 mM NaHCO3, 0.02% (v/v) rabbit anti-chicken IgG immunoglobulin antibody (Z247, Dako, Glostrup, Denmark) was coupled to CNBr-activated-Sepharose 4B (Amersham Pharmacia Biotech) at a concentration of 3.3 mg of antibody/ml of gel. Rabbit anti-chicken IgG antibody (whole molecule) (C-2888, Sigma); alkaline phosphatase-con-
jugated goat anti-rabbit IgG antibody (whole molecule) (A-8025, Sigma); alkaline phosphatase-labeled avidin (A-2527, Sigma); and p-nitrophenyl phosphate, disodium salt (Roche Molecular Biochemicals) were used. Gelatin was from Difco (control 683487). Bovine serum albumin was from Sigma (A-7639). Maltose-TSK gel was prepared by coupling 100 mg of divinylsulfonyl-methyl-Sepharose (75% cross-linked) (14985, Merck, Darmstadt, Germany) (24). Mannan was prepared from *Saccharomyces cerevisiae* (25).

**Purification of bMFAP4 and Bovine Lung SP-D**—Bovine lungs were obtained from a local abattoir. The lungs were washed at 4 °C with TBS containing enzyme inhibitors (5 mM iodoacetamide, 5 mM cyclohexylcarboxyl acid, Kabi Pharmacia, Sweden), 5 mM EDTA, 10 units/ml Trasylol (aprotinin, Bayer, Leverkusen, Germany). The lung washings (500 ml) were clarified by centrifugation at 10,000 × g for 15 min at 4 °C; then calcium was added to 25 mM, and the pH was adjusted to 7.4. After another centrifugation (10,000 × g for 30 min) the supernatant was passed through a 50-ml maltose-TSK column. The maltose-TSK column was washed extensively with TBS containing enzyme inhibitors and 25 mM CaCl$_2$ and bound SP-D was eluted with TBS/10 mM CaCl$_2$ and 100 mM maltose. After another wash with TBS/10 mM CaCl$_2$, bound bMFAP4 was eluted with TBS/10 mM EDTA. The eluate containing bMFAP4 was passed separately through a 10-ml rabbit anti-bovine Ig-Sepharose column to remove antibodies. Fragments of bMFAP4 were further purified on a Superose 6 Prepgrade column (125 × 2.6 cm, Amersham Pharmacia Biotech) at a flow rate of 30 ml/h.

The apparent molecular weight of bMFAP4 was estimated by gel permeation chromatography on a Superose 12 column (30 × 1 cm, Amersham Pharmacia Biotech) at a flow rate of 24 ml/h. The bMFAP4 sample was concentrated using Centricon concentrators (Amicon, Beverly, MA), and a 200-μl sample containing approximately 25 μg/ml was then applied to the column with TBS/10 mM EDTA as running buffer. Blue dextran, fibronectin, and rabbit IgG were used to calibrate the column.

The purification of bMFAP4 and SP-D was monitored by SDS-PAGE, and the amount of protein in the fractions was estimated by their UV-absorption at 280 nm on the assumption that ε$_{280}$ for bMFAP4 and SP-D was 1.0.

**Expression of Recombinant Rat SP-D, Recombinant Rat SP-Dala72, and Human SP-D Neck-CRD**—Recombinant rat SP-D (rSP-D) and recombinant rat SP-Dala72 (rrSP-Dala72) were expressed in Chinese hamster ovary-K1 cells using the pE14E4 transfection vector (26). Human SP-D (hSP-D) neck-CRD was expressed using the pET32 expression system (Novagen). Primers used for PCR amplification were 5’-CGGAATTCCTCAGAACTCGCAGA-3’ (sense) and 5’-CCGCGGATCCGGATTGAAGGGGG-3’ (antisense) using a full-length cDNA as template. The PCR product was inserted using the BamHI and EcoRI cloning sites. *Escherichia coli* strain BL21-de3 was transformed by electroporation, and the pET32-rSP-D construct was sequenced before expression. Purification of the expressed protein was performed on nickel-nitrilotriacetic acid resin followed by enterokinase cleavage and removal of dNTP, 20 ng of cDNA template, and 2 units of Taq polymerase (Life Technologies, Inc.) in the manufacturer’s buffer. Thirty cycles of PCR amplification were performed with a denaturing temperature at 94 °C for 45 s, annealing at 55 °C for 45 s, and allowing extension at 72 °C for 1 min. The first denaturation cycle was prolonged to 2 min, and the final extension after the expected 34 PCR cycles was prolonged to 5 min. The PCR product was obtained and ligated into pCRII (Invitrogen BV). Plasmid DNA was prepared, digested with *XbaI* and *SalI*, and separated on an agarose gel. After electrophoresis, the MFAP4 cDNA fragment was cut from the gel, extracted with the Sephasag Bandprep kit (Amersham Pharmacia Biotech), and then ligated into pTrxFus (Invitrogen), which had previously been digested with *SalI* and *XbaI*.

The construct (pSP-Etrx) was transformed into *E. coli* strain G7124, and plasmid DNA was isolated and sequenced to confirm that the subcloning had been successful.

A single colony carrying pSP-Etrx was inoculated into 3 ml of RMG-Amp medium containing 1X M9 salts, 2% casamino acids, 0.5% glucose, and 0.5% L-lysine (M9 Complete). Prat plates were prepared with TSB containing 0.5% glucose, 2 mM MgCl$_2$, and 100 μg/ml ampicillin, and 5 ml of culture were seeded onto plates with a TSB overlay containing 5 mM CaCl$_2$, 100 mM maltose, and 0.5% glucose. Fifty ml of induction medium (1X M9 salts, 0.2% casamino acids, 0.5% glucose, 1 mM MgCl$_2$, and 100 μg/ml ampicillin) were inoculated with 3 ml of culture and incubated at 30 °C until the absorbance at 550 nm was ~0.5. Protein expression was induced by adding 100 μg/ml L-tryptophan, and the culture was allowed to grow for 4 h at 30 °C before the MFAP4 was harvested. Cells expressing high levels of bMFAP4 were resuspended in 2.5 ml of TSB (50 mM NaCl, 100 mM Tris-HCl, 1 mM EDTA, 0.5% SDS, pH 7.0) containing 0.1 mM phenylmethylsulfonyl fluoride and lysed by three cycles of sonication, rapid freezing, and thawing. Centrifugation of the sample at 12,000 × g gave a clear supernatant containing 8 mg/ml soluble protein. Protein (20 mg) was applied to a column of Thiobond™ resin (Invitrogen) and eluted with a gradient of 2-mercaptoethanol from 1 to 500 mM in TSB. Fractions were examined for recombinant protein by SDS-PAGE.

As a control, the fusion partner, a 12-kDa thioredoxin peptide, was expressed in *E. coli* using the plasmid pTrxFus. Thioredoxin control peptide was purified by osmotic shock according to the manufacturer’s protocol (27).

**Biotinylation—** Purified bMFAP4 and rhMFAP4 were labeled with biotin (28). The proteins were dialyzed against phosphate-buffered saline adjusted to pH 8.5 with 3% (w/v) Na$_2$CO$_3$ and biotin-N-hydroxysuccinimide ester (Sigma H-1759, 40 mg/ml in dimethyl sulfoxide) was added at 0.17 mg/ml protein. The mixture was incubated for 4 h at room temperature and dialyzed against TBS/E. The labeled bMFAP4 and rhMFAP4 were analyzed by SDS-PAGE and Western blotting.

**SDS-PAGE and Western Blotting—** Electrophoresis was performed on 4–20% (w/v) polyacrylamide gradient gels in a discontinuous buffer system (29). Samples were reduced by heating at 100 °C for 1 min in 60 mM dithiothreitol, 1.5% (w/v) SDS, 5% glycerol, 0.02% bromophenol blue, 0.1% Triton X-100, and 8.0% pH, and alkylated by the addition of iodoacetamide to a concentration of 140 mM. Unreduced samples were heated for 1 min in sample buffer with 40 mM iodoacetamide followed by the addition of further iodoacetamide to a concentration of 180 mM. Protein bands were detected by silver staining (30).

Separated proteins were electrophoresed (31) onto polyvinylidene difluoride membranes (Immobil-P, Bedford, MA) with 25% (v/v) ethanol being substituted for methanol in the buffer. The paper was cut in 2-mm strips and incubated with primary chicken antibodies overnight, followed by incubation with a biotin-phosphate-coupled goat anti-rabbit IgG for 1 h. The antibodies were diluted in high salt TBS (TBS containing 0.5 mM NaCl and 0.05% Tween). Control strips were incubated in high salt TBS or normal chicken IgG purified from chicken egg yolk and diluted in high salt TBS. When biotinylated bMFAP4 and biotinylated rhMFAP4 were blotted, the strips were incubated with alkaline phosphatase-coupled avidin for 1 h. The proteins were treated with nitrocellulose and washed with phosphate-buffered saline containing 0.5% Tween, 0.1% Triton X-100, and 10% isopropanol. The membranes were cut with a scalpel and transferred to immunoblotting buffer, which consisted of 0.5% Tween, 0.1% BSA, 5% skim milk, 0.01% sodium azide, and 100 mM Tris-HCl, 1 mM EDTA, 0.5% SDS, pH 7.4. Bands were visualized with 10% sucrose, 0.5% BSA, 0.02% diaminobenzidine, and 0.02% hydrogen peroxide as a chromogen (32).

**Preparation of Anti-bMFAP4 Antibodies—** Antibodies against bMFAP4 were raised in chickens by subcutaneous immunization with an emulsified mixture of equal volumes of bMFAP4 (19 μg) and Freund’s complete adjuvant (Statens Serum Institut, Copenhagen, Denmark). The chickens were boosted 1 and 3 months after the initial immunization with the same amount of antigen in Freund’s complete adjuvant.

**Purification of IgG from Chicken Egg Yolk—** Egg yolk (15 ml) was suspended in 15 ml of TBS and 60 ml of 0.06 M sodium acetate buffer, pH 4.0, after which 2 ml of caprylic acid (Statens Serum Institut) were added. After storing for 30 min at room temperature the mixture was centrifuged (10,000 × g for 25 min at 4 °C), and the pellet and floating
globular proteins were used as markers (Fig. 2). Approximately
and showed an apparent molecular mass of 250 kDa when
analyzed by gel permeation chromatography on Superose 12
migrates as a single band of 66 kDa. Purified bMFAP4 was
and a minor band of 70 kDa, whereas unreduced bMFAP4
2
SP-D in the maltose eluate. Fig. 1 shows SDS-PAGE analysis of
purifications a proportion of bMFAP4 was eluted together with
bodies on a rabbit anti-bovine Ig-Sepharose column. In some

Enzyme-linked Binding Assay—Microtiter plates (Polsysorb, Nalgé-
Nunc International, Kamstrup, Denmark) were coated with purified
SP-D, recombinant rat SP-D, SP-Dala72, neck-CRD SP-D, gelatin, bo-
vine serum albumin, or mannan (1 μg/ml in coating buffer) for 2 h at
room temperature. When collagenase-digested SP-D was used, the
plates were coated with 10 μg of protein/ml. All incubations were
carried out in a volume of 100 μl/well at room temperature in a moist
chamber. The plates were washed three times with TBS/Tw and
blocked with TBS/Tw containing either 5 mM CaCl₂ or 10 mM EDTA for
30 min. The plates were then incubated overnight with dilutions of
biotinylated bMFAP4 or biotinylated rhMFAP4 in TBS/Tw containing
0.1% bovine serum albumin and either 5 mM CaCl₂ or 10 mM EDTA.
This and the following steps were carried out on a shaking platform.
Between all the following steps the plates were washed three times in
TBS/Tw containing either 5 mM CaCl₂ or 10 mM EDTA. In the inhibition
assay the binding of biotinylated bMFAP4 to SP-D was inhibited by
unlabeled bMFAP4 at various concentrations in the presence of 5 mM
CaCl₂. After washing, the plates were incubated with alkaline phos-
phatase-coupled avidin diluted 1/1000 in TBS/Tw buffer containing
either 5 mM CaCl₂ or 10 mM EDTA. After a final wash, the bound
enzyme was estimated by adding p-nitrophenylphosphate, disodium
salt at 1 mg/ml in substrate buffer. The absorbance of the wells was
read at 405 nm by means of a multichannel spectrophotometer (EAR
400 FT; SLT-LabInstruments, Innsbruck, Austria).

Amino Acid Sequencing and Amino Acid Analysis—The procedures
were as described (32). Amino acid analysis was performed directly on
purified bMFAP4 in an Applied Biosystems 420A amino acid analyzer
(Perkin-Elmer, Applied Biosystems Division). For N-terminal and pep-
tide sequencing, purified bMFAP4 was run on SDS-PAGE and electro-
blotted onto polyvinylidene difluoride membranes prior to detection
with Ponceau-S dye. The bMFAP4 band was excised from the blot and
blotted onto polyvinylidene difluoride membranes prior to detection
with Ponceau-S dye. The bMFAP4 band was excised from the blot and

RESULTS

Identification and Purification of bMFAP4—During the pur-
ification of bovine SP-D a molecule with a molecular mass of
36 kDa was observed on SDS-PAGE in the reduced state. This
molecule was subsequently identified by N-terminal sequenc-
ing as the homolog of bMFAP4. The 10,000 × g supernatant
from bovine lung washings was applied to a maltose-TSK col-
umn in the presence of 5 mM CaCl₂, and after eluting SP-D with
100 mM maltose, bMFAP4 was eluted with 10 mM EDTA. Bo-
vine MFAP4 and SP-D were cleared of anti-carbohydrate anti-
odies on a rabbit anti-bovine Ig-Sepharose column. In some
purifications a proportion of bMFAP4 was eluted together with
SP-D in the maltose eluate. Fig. 1 shows SDS-PAGE analysis of
purified bMFAP4 in the reduced (lane 1) and unreduced (lane
2) state. Reduced bMFAP4 migrates as a major band of 36 kDa
and a minor band of 70 kDa, whereas unreduced bMFAP4
migrates as a single band of 66 kDa. Purified bMFAP4 was
analyzed by gel permeation chromatography on Superose 12
and showed an apparent molecular mass of 250 kDa when
globular proteins were used as markers (Fig. 2). Approximately
800 μg of bMFAP4 (estimated by E₂₈₀) was obtained from 1
liter of lung washings.

Deglycosylation of bMFAP4—N-linked glycosylation was es-
timated by means of N-glycosidase F digestion, and the product
was analyzed by SDS-PAGE and Western blotting. Western
blotting was necessary because of the identical molecular mass

of N-glycosidase F and deglycosylated bMFAP4. Fig. 3A
shows bMFAP4 in the reduced state before (lane 1) and after (lane
2) treatment with N-glycosidase F. The molecular mass of
bMFAP4 is reduced from 36 to 33 kDa upon deglycosylation.
The specificity of the polyclonal anti-bMFAP4 chicken antibo-
dies was analyzed by Western blotting of crude bovine lung
washings containing bMFAP4 in the reduced state (Fig. 3B). A major specific band is seen at a position corresponding to a molecular mass of 36 kDa (lane 2).

Amino Acid Sequencing and Amino Acid Analysis—The N-terminal amino acid sequence and amino acid sequences of different proteolytic fragments obtained by tryptic digestion of bMFAP4 are shown in Fig. 4, A and B. The N terminus of bMFAP4 showed homology to a 36-kDa microfibril-associated protein (MAP) found in bovine (34) and porcine aorta (33) and to human MFAP4 (23). An Arg-Gly-Asp (RGD) sequence is conserved in the N termini of these proteins. This sequence motif is often associated with cell adhesive activity and is the ligand motif for cell surface integrins (37, 38).

cDNA Cloning and Expression of rhMFAP4—A full-length cDNA clone of MFAP4 was obtained from a human cDNA kidney library and showed 99.8% identity with the published sequence (23) with a single base shift (A to G) changing the amino acid at position 124 from an Asp to a Gly. The coding region spanned 255 amino acids with an N-terminal region of 16 amino acids containing an Arg-Gly-Asp sequence and one cysteine residue. The N-terminal region was followed by a single fibrinogen-like domain of 239 amino acids showing high homology to fibrinogen domains found in human ficolin (36) and P35 (10) (Fig. 4C). A potential calcium binding site is marked with a dotted line, and the residues involved in calcium binding, as deduced from the crystal structure of the fibrinogen γ chain, are shown in open boxes. The potential integrin binding site is indicated with a bold dashed line. Gaps in the alignment are shown by a light dash.

Reduced state, and unordered rhMFAP4 shows the same mobility (data not shown), indicating that the recombinant molecule fails to form disulfide bondings.

Binding Specificity—In the presence of 5 mM CaCl2, rhMFAP4 and bMFAP4 bound to microtiter plates coated with bovine SP-D (Fig. 6). The binding was concentration-dependent and inhibited by 10 mM EDTA. No binding of bMFAP4 was seen if SP-D was digested with collagenase, indicating that bMFAP4 binds to the collagen region of SP-D (Fig. 7A). Purified SP-D is shown in Fig. 7C, lane 1, and collagenase-treated SP-D is shown in lane 2. The 43-kDa band seen in lane 1 has disappeared and a 20-kDa band corresponding to the neck-CRD region of SP-D has appeared. The additional bands seen from 60 to 100 kDa originate from the collagenase preparation. bMFAP4 and rhMFAP4 also showed calcium-dependent binding to gelatin supporting the idea that MFAP4 binds to the collagen region of SP-D (Fig. 7B). rhMFAP4 bound equally well to rrSP-D and rrSP-Dala72 in a dose-dependent manner, whereas no binding was seen to hSP-D neck-CRD (Fig. 8). In the same experiment no binding was seen to bovine serum albumin. The binding between MFAP4 and solid-phase SP-D was not inhibited by SP-D in solution at a concentration of 25 μg/ml (data not shown).
Fig. 9 shows that bMFAP4 and rhMFAP4 also bound to microtiter plates coated with mannan. This binding was concentration- and calcium-dependent and took place at physiological ionic strength. The binding was partially inhibited by 100 mM maltose.

The binding of biotinylated bMFAP4 or rhMFAP4 to SP-D could be inhibited by unlabeled bMFAP4 or rhMFAP4, respectively, demonstrating that the biotinylation had not radically altered the binding properties of bMFAP4 or rhMFAP4 (data not shown).

DISCUSSION

The present report describes the identification, purification, and characterization of an SP-D-binding molecule from bovine lung washings. The protein was identified as the bovine homolog of human MFAP4 and was termed bMFAP4. Human recombinant MFAP4 and bMFAP4 showed calcium-dependent binding to the collagen structure of SP-D and to mannan.

On SDS-PAGE bMFAP4 showed a molecular mass of 36 kDa in the reduced state and 66 kDa in the unreduced state. A minor band at 70 kDa seen on SDS-PAGE in the reduced state was probably because of incomplete disruption of an interchain disulfide bond. This band pattern has also been observed for the homologue porcine 36-kDa MAP (33). The apparent molecular mass of native bMFAP4 was estimated as 250 kDa on gel permeation chromatography. N-linked glycosylation was demonstrated by digestion with N-glycosidase F, which reduced the molecular mass to 33 kDa. These data suggest that bMFAP4 is a disulfide-linked homodimeric glycoprotein with a molecular mass of 66 kDa, organized into a higher oligomeric form via noncovalent interactions.

The N-terminal amino acid sequence of bMFAP4 showed a high degree of homology with hMFAP4 with 10 of 14 amino acid residues being conserved, including the RGD motif and the cysteine at position 10 (23). The N-terminal amino acid sequence also showed homology with the microfibril-associated glycoproteins (36-kDa MAP) found in the bovine and porcine aorta. On SDS-PAGE the migration pattern of reduced and unreduced bovine 36-kDa MAP was similar to the migration pattern of reduced and unreduced bMFAP4 (34), but the N-terminal amino acid sequence of 36-kDa MAP deviated at one position from bMFAP4. Possibly two closely related forms of bMFAP4 exist.

The short peptide sequence RGD is conserved in the 36-kDa aorta MAP, hMFAP4, and bMFAP4. This sequence was initially seen in fibronectin (37) and is the ligand motif for cell surface integrins. The RGD motif is found in many other proteins involved in cell adhesive activity (38). These include the tenascins, which form a family of large and complex extracellular matrix proteins (43). They are believed to be involved in processes of tissue formation and remodeling. The tenascins also contain a C-terminal fibrinogen-like domain, which in human (44) and porcine tenascins (45) shows a 52–54% sequence identity to hMFAP4 (23). The cell adhesive activity of
the tenascins has also been located to the C-terminal fibrinogen-like domain (46). An internal peptide sequence from the fibrinogen \( \beta \) chain (GWTVFQKRLDGSV) has been shown to be involved in binding to the leukocyte integrin Mac-1 (41). This peptide sequence is highly conserved between the fibrinogen \( \beta \) chain and hMFAP4. Thus two potential cell adhesive motifs are found in hMFAP4.

The fibrinogen-like domain of hMFAP4 reveals a 48–50% sequence identity to the fibrinogen-like domains of the human ficolins. Two types of ficolin are found in man; P35 or L-ficolin is a plasma protein synthesized by the liver, and M-ficolin is mainly synthesized by monocytes and can be detected on the monocyte surface. The ficolins are composed of collagen-like regions attached to fibrinogen-like domains. Via its fibrinogen-like domain, P35 can bind to the glycosylated surfaces of pathogens and enhance phagocytosis of these pathogens by neutrophils, and P35 also interacts in a calcium-dependent manner with mannan (10). Terbium fluorescence studies localized a calcium binding site on the human fibrinogen \( \gamma \) chain, which corresponds to residues 162–187 in hMFAP4 (40), and the crystal structure of the human fibrinogen \( \gamma \) domain revealed a short calcium-binding loop in the same region with the four residues Asp-171, Asp-173, Phe-175, and Gly-177 as ligands for the calcium ion (39). Asp-171 and Asp-173 are highly conserved in all fibrinogen domains, whereas the residues at positions 175 and 177 vary considerably. Three of the four calcium ligand residues are conserved in hMFAP4, Gly-177 being substituted by Gln in hMFAP4. Calcium binding site 2 of the C-type lectin CRD is the center of calcium-dependent carbohydrate recognition. Five residues are responsible for this interaction, three of which are highly conserved in all C-type CRDs, whereas the remaining two vary and determine the carbohydrate specificity of the C-type lectin (47). Although the structure of the calcium-binding loop of fibrinogen differs considerably from that of the C-type CRD, it is tempting to speculate that residues 175 and 177 might determine the calcium-dependent ligand specificity of the fibrinogen domain.

Both bMFAP4 and rhMFAP4 bind in a calcium-dependent manner to SP-D at physiological ionic strength. bMFAP4 does not interact with collagenase-digested bSP-D, and rhMFAP4 does not interact with a recombinant form of human SP-D lacking the collagen domain. These results indicate that the interaction is mediated by the collagen region of SP-D and this is further supported by the finding that both bMFAP4 and rhMFAP4 bind calcium independently to collagen in the form of gelatin. The binding between MFAP4 and solid-phase SP-D was not inhibited by SP-D in solution at a concentration of 25 \( \mu \)g/ml (data not shown). This could mean that conformational changes induced by partial denaturation of the collagen region of SP-D are needed before binding between SP-D and MFAP4 can take place. Such conformational changes may take place during inflammation.

Both rhMFAP4 and bMFAP4 bind to mannan. This interaction is partly inhibited by maltose and totally inhibited by EDTA. Bovine MFAP4 bound to the maltose-TSK column in the presence of calcium, but only trace amounts of bMFAP4 were eluted with 100 mM maltose, whereas the rest was subsequently eluted with EDTA. In fact bMFAP4 also bound to a nonderivatized TSK column (data not shown), suggesting that...
other forces than the lectin-carbohydrate interaction are involved in the calcium-dependent binding of bMFAP4 to the maltose-TSK column.

The lectin activity of MFAP4 raised the possibility that the interaction takes place via the N-linked carbohydrate located in the collagen region of SP-D at position Asn-70. We therefore compared the binding between rhMFAP4 and rrSP-Dala72 and wild-type rrSP-D. rrSP-Dala72 has substituted a Ser to Ala at the recognition site. The two forms of rat SP-D bound equally well to rhMFAP4, and therefore the N-linked carbohydrate does not influence the interaction between SP-D and MFAP4.

Bovine and porcine MAP were earlier described as extracellular matrix proteins purified from aortic tissue (33). In humans, deletions of the MFAP4 gene have been correlated with Smith-Magenis syndrome, which is characterized by multiple congenital anomalies and mental retardation. We have now shown that bMFAP4 is present in lung washings as a soluble protein. Other extracellular matrix proteins, such as fibronectin and fibrin, are also known to be present both in the extracellular matrix and in various body fluids.

The properties of MFAP4 show that it has the potential of opsonizing microorganisms on its own further differentiation of the phagocytes (50). Finally, MFAP4 is known to be present both in the extracellular matrix and in various body fluids.

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