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A novel calcium-binding protein in amniotic fluid, CAAF1: its molecular cloning and tissue distribution

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SUMMARY

We found by using a $^{45}Ca^{2+}$ overlay technique a large amount of Ca^{2+}-binding activity in bovine amniotic fluid from which a novel calcium-binding protein (CaBP) was purified and is referred to as CAAF1 (calcium-binding protein in amniotic fluid-1), with an apparent molecular mass of 8 kDa determined by *N*-tris(hydroxymethyl)-methylglycine/SDS-PAGE. It was structurally homologous with MRP/calgranulin proteins (MRP8/calgranulin A and MRP14/calgranulin B), members of the S100 protein family, which are abundantly found in the cytoplasm of granulocytes and macrophages. CAAF1 lacked the predicted signal peptide sequence, which is consistent with other CaBPs. The tissue and cellular distribution of CAAF1 was determined by monoclonal antibodies developed against this protein. Its immunoreactivity was found in squamous epithelial cells, neutrophils, and some macrophages throughout the fetal body. An especially characteristic staining pattern was obtained in the squamous epithelium, including that of the esophagus, skin and amnion: CAAF1 was detected in the suprabasal squamous epithelial cells undergoing differentiation, but not in the cells in the proliferating basal layer. Northern blot analysis also showed that CAAF1 mRNA was highly expressed in bovine fetal esophagus and skin. On the other hand, our ELISA studies showed that CAAF1 protein was present in amniotic fluid at a concentration of about 120 nM, which was over 30 times as high as that in the fetal serum. These results suggested that CAAF1 is one of the stage-specific proteins in the differentiation of squamous epithelial cells, and that CAAF1 is preferentially produced by fetal squamous epithelial cells, including epidermal keratinocytes and amniotic epithelial cells, and it is stored in the amniotic fluid during embryogenesis.

Key words: calcium-binding protein, S100 protein family, amniotic fluid, squamous epithelial cell differentiation, embryogenesis

INTRODUCTION

An increase in the intracellular concentration of Ca^{2+} caused by stimulation by growth factors or peptide hormones can trigger such various responses as cellular motility, proliferation, secretion, metabolism and expression of the genes involved in cell differentiation. Transmission of the Ca^{2+} signals requires a variety of calcium-binding proteins (CaBPs), which enable Ca^{2+} to act as a triggering second-messenger element that induces conformational changes in effector molecules (Davis, 1992; Clapham, 1995).

On the other hand, the targets of the signal of increased Ca^{2+} differ, dependent upon the cell-specific information (Kligman and Hilt, 1988). The CaBPs include the S100 protein family of small CaBPs, which have two Ca^{2+}-binding sites in one molecule with nearly a 10 kDa molecular mass. They are considered to be expressed in a cell-type specific fashion and, therefore, there is a possibility that the S100 proteins are excellent candidates to function as cell-type specific transducers of Ca^{2+} signaling.

We thought it worthwhile to investigate the existence and function of CaBPs for understanding the mechanisms of cell growth and differentiation, and we searched various materials for a clue to these proteins in some materials. Unexpectedly we found a large amount of Ca^{2+}-binding activity in bovine amniotic fluid by using a $^{45}Ca^{2+}$ overlay technique, and isolated and characterized one of the substances responsible for this activity, referred to as CAAF1 (calcium-binding protein in amniotic fluid-1). It was a novel CaBP structurally homologous with MRP/calgranulin proteins (MRP8/calgranulin A and MRP14/calgranulin B; synonyms of MRP8 and MRP14 are calgranulins A and B, L1 light and heavy chain, respectively), members of the S100 protein family.

While there is no information on CaBPs in amniotic fluid, there are some reports of the S100 protein family in some body fluids. For example, S100β, which might be released from pituitary cells and glial cells, is found in cerebrospinal fluid (Ishikawa et al., 1983; Shashoua et al., 1984; Suzuki et al., 1987). Many patients with infectious disorders or cystic fibrosis have elevated plasma levels of MRP8 and MRP14,

which have also been referred to as L1 complex or CF antigen (CFAg) (Dale et al., 1983; van Heyningen et al., 1985; Dorin et al., 1987; Odink et al., 1987; Andersson et al., 1988). In spite of all the reports on the presence of extracellular CaBPs, their possible physiological functions remain elusive.

In this paper, we describe the amino acid and complete cDNA sequences of CAAF1, and its tissue and cellular distribution determined by immunohistochemistry, northern blot analysis, and ELISA. We also discuss where CAAF1 is derived from and its biological functions.

MATERIALS AND METHODS

Materials

Amniotic fluid and fetal tissues from cows in the fourth month of pregnancy were purchased from Mitsubishi Fine Chemical (Tokyo, Japan). Lysylendopeptidase (EC 3.4.21.50) was obtained from Seikagaku Industrial (Tokyo), and endoproteinase Glu-C (EC 3.4.21.19) from Boehringer GmbH (Mannheim, Germany). T_4 DNA polymerase and Taq polymerase (Ampli Taq) were obtained from Takara Shuzo Co., Ltd (Otsu, Japan). Restriction endonucleases and other modifying enzyme were purchased from Toyobo Co., Ltd (Osaka, Japan). Oligonucleotides were synthesized on a Model 392A DNA synthesizer (Applied Biosystems Inc. Foster City, CA). $^{45}Ca^{2+}$ (1 GBq/mg Ca) was from DuPont, NEN Research Products, Boston, MA, and [α-^{32}P]dCTP (110 TBq/mmol) was from Amersham International (Buckinghamshire, England). All other chemicals and reagents were of analytical grade and obtained from commercial suppliers.

Detection of calcium-binding proteins in amniotic fluid

A 5 ml sample of amniotic fluid was applied to an ODS cartridge (Sep-PaK C_{18}; Millipore Corp., Bedford, MA). After the column was washed, the retained fraction was eluted with 80% acetonitrile in 0.1% trifluoroacetic acid, and used for detection after lyophilization. The samples were dissolved in 100 μl of distilled water. A fraction of 20 μl was separated by *N*-tris(hydroxymethyl)methylglycine (Tricine)/SDS-PAGE according to Schagger and von Jagow (1987), and the proteins were transferred to Immobilon (Millipore) by the semidry blot method. Ca^{2+}-binding activity was detected by $^{45}Ca^{2+}$ autoradiography according to the method of Maruyama et al. (1984).

Purification of calcium-binding proteins in bovine amniotic fluid and protein sequencing

Bovine CaBPs were isolated from amniotic fluid as follows: amniotic fluid (3,000 ml) was adjusted to pH 3.0 with acetic acid, and centrifuged for 10 minutes at 9,000 ***g*** at 4°C. After centrifugation, the supernatant was filtered through filter paper (no. 5A; Advantec, Tokyo), and applied to a 50 ml S-Sepharose fast flow ion exchange column (2.6 cm × 10 cm; Pharmacia, Uppsala, Sweden) equilibrated with 1.0 M acetic acid. The column was washed with 20 vols of 1.0 M acetic acid, and a linear gradient of CH_3COONH_4 (0-1.0 M) at a flow rate of 4 ml/minute was used to elute proteins. Samples (20 μl) of the fractions were analyzed by Tricine/SDS-PAGE and the $^{45}Ca^{2+}$ overlay technique described above. The fractions with Ca^{2+}-binding activity were collected and lyophilized. These samples were reconstituted with 5 ml of 1.0 M acetic acid, and chromatographed on a Sephadex-G75 fine column (1.5 cm × 90 cm; Pharmacia) equilibrated with 1.0 M acetic acid. The active fractions from the gel filtration step were directly subjected to reverse-phase HPLC on a TSK phenyl-5PW column (4.6 mm × 75 mm; Tosoh) equilibrated with 0.1% (v/v) trifluoroacetic acid, and eluted with a linear gradient from 25 to 42% (v/v) acetonitrile at a flow rate of 1 ml/minute. The final purification of CaBPs was carried out on a TSK ODS-120T reverse-phase HPLC column (4.6 mm × 150 mm; Tosoh), eluted as above. Samples (20 μl) were analyzed by Tricine/SDS-PAGE, and the purity was determined with a silver staining kit (Wako Pure Chemical Industries, Ltd, Tokyo). The concentration of purified CAAF1 was measured with a BCA protein assay kit with bovine serum albumin (BSA) as calibrator (Smith et al., 1985). This grade of CAAF1 was used as a calibrator for a sandwich ELISA.

Purified CAAF1 (5 μg) was digested with lysylendopeptidase (EC 3.4.21.50) or endoproteinase Glu-C (EC 3.4.21.19) at an enzyme-to-substrate ratio of 1:20 (w/w) in 50 μl of 0.1 M Tris buffer, pH 9.0, or 0.1 M ammonium buffer, pH 4.0. After overnight incubation at 37°C, the resulting peptides were purified by a C_8 microbore reverse phase column (Model 130A; Applied Biosystems) The column was developed with a linear gradient of acetonitrile (0-80%) in 0.1% CF_3COOH. The effluent was monitored at 210 nm. The amino acid sequence was determined with an automated protein sequencer (Model 477A; Applied Biosystems).

Isolation of CAAF1 cDNA clones and DNA sequencing

An authentic cDNA probe of CAAF1 was isolated by RNA-PCR amplification. First strand cDNA was synthesized from 200 ng of poly(A)$^+$ RNA of bovine fetal esophageal tissue, with MMLV reverse transcriptase and random hexamerprimers using a GeneAmp™ RNA-PCR kit (Perkin-Elmer Corp., Norwalk, CT) according to the manufacturer's instructions. After first strand cDNA was synthesized, PCR amplification was performed by using the whole first strand cDNA and 1 nmole of each of the degenerate primers, P7S1 (sense) and P7A1 (antisense), which were designed according to the peptide sequence of CAAF1, LEDHLEG and AHIDIHK, respectively. The oligonucleotide mixtures were synthesized by a DNA/RNA synthesizer (Model 392, Applied Biosystems) as follows:

P7S1 (sense): 5′ TT(A/C/G/T)GA(A/G)GA(C/T)CA(C/T)(C/T)-T(A/C/G/T)GA(A/G)GG 3′.
P7A1 (antisense): 5′ TT(A/G)TG(A/G/T)AT(A/G)TC(A/G/T)-AT(A/G)TG(A/C/G/T)GC 3′.

The PCR reaction was cycled 35 times (94°C for 1 minute, 48°C for 2 minutes, and 72°C for 2 minutes). The purified PCR product of the expected size (263 bp) was ligated into *Sma*I-digested pTZ (Pharmacia). The *Pst*I and *Xba*I cleaved fragments from the single clone, whose deduced amino acid sequence corresponded to the CAAF1 amino acid sequence, was used as the authentic cDNA probe of CAAF1.

Double-stranded cDNA for the construction of a cDNA library was prepared from 5 μg of poly(A)$^+$ RNA of bovine fetal esophageal tissue with MMLV reverse transcriptase and oligo (dT)12-18 primers using a TimeSaver™ cDNA synthesis kit (Pharmacia) according to the manufacturer's manual, and the library was constructed in λgt11. About 2×10^5 plaques from the bovine fetal esophageal λgt11 cDNA library were screened with the cDNA probe labeled by the multi-priming method using random hexamer primers. The insert DNA of the positive clones was isolated by PCR amplification using the two λgt11 primers flanking the cDNA insert. The amplified insert DNA was subcloned into the pTZ vector, and they were subjected to DNA sequencing.

Nucleotide and amino acid sequences were determined with Genetyx™ software packages. The sequence homology searches were carried out by using the FASTA and TBLASTX computer programs of the Genetics Computer Group.

Production of monoclonal antibodies

Female Balb/c mice, 8-10 weeks old, were immunized with the purified CAAF1 protein from bovine amniotic fluid. The mouse splenocytes were fused with SP2/0-Ag14 myeloma cells. Hybridoma supernatants were initially screened for relevant mAbs against CAAF1 by an RIA, which was performed as follows: purified rabbit anti-mouse IgG was bound to the bottom of the wells of a 96-well

plate (Nunc, Roskilde, Denmark) by adding 50 μl of the antibody solution (10 μg/ml) to each well. The wells were blocked with 3% BSA in Hepes binding buffer (HBB) (128 mM NaCl, 5 mM KCl, 5 mM $MgSO_4$, 1.2 mM $CaCl_2$, 50 mM Hepes, pH 7.5) for 1 hour at room temperature. After three washings with 0.05% Tween-20 in HBB, 50 μl quantities of hybridoma supernatants were added to the wells and the plate was incubated for 1 hour at room temperature. The plate was again washed three times with 0.05% Tween-20 in HBB and incubated with ^{125}I labeled CAAF1 (50,000 cpm) in 50 μl of HBB containing 3% BSA. The RIA plate was washed four times and the bound ^{125}I labeled CAAF1 was eluted by adding 100 μl of 10% acetic acid (v/v). The eluant was counted with a gamma counter. CAAF1 was iodinated with $Na^{125}I$ using Iodogen (Pierce Chemical Co., Rockford). The specificity of the monoclonal antibodies was also determined by an immunoblot method. The immunoglobulin subclasses of mAbs were determined with a mouse monoclonal antibody isotyping kit (Amersham).

The cloned hybridoma cells were injected into mice, the ascitic fluids were collected and the monoclonal antibodies were purified by affinity chromatography using Protein A-Sepharose CL-4B (Pharmacia).

ELISA and immunohistochemistry

For the quantification of CAAF1 in bovine amniotic fluid, a sandwich assay was performed with mAb 22-5 and biotinylated mAb 25-25. The purified GFD/CAAF1/25-25 was biotinylated with sulfosuccinimidyl 6-(biotinamido) hexanate (NHS-LC-biotin) (Pierce). Biotin-ylated mAbs were purified by gel filtration chromatography on a Sephadex G25 column (Pharmacia). The wells of 96-well microtiter plates were coated with mAb 22-5 by incubation overnight with 100 μl of the immunoglobulin solution (10 μg/ml) in 50 mM sodium bicarbonate, pH 9.4, at 4°C. After the reaction was blocked with 10 mg of ovalbumin per ml in HBS (0.15 M NaCl, 20 mM Hepes, pH 7.4), the plates were incubated for 1 hour with 100 μl of 5 mg/ml ovalbumin and 0.05% Tween-20 in HBS (OVA-T-HBS), containing appropriately diluted samples or various amounts of CAAF1. After being washed with HBS containing 0.05% Tween-20, the plates were incubated for 1 hour with 100 μl of the biotinylated mAb 25-25 at a concentration of 1 μg/ml in OVA-T-HBS. After further washing, the plates were incubated for 1 hour with 100 μl of a 5,000-fold dilution of streptavidin-peroxidase conjugate (Vector Laboratories, Burlingham, CA) in OVA-T-HBS. The reactions were allowed to proceed at room temperature. Thereafter, washing was repeated and substrate buffer consisting of 0.4 μg of *o*-phenylenediamine per ml and 0.006% H_2O_2 in 0.1 M citrate buffer pH 5.6, was added. After the enzyme reaction was stopped with 100 μl of 1 M H_2SO_4, the absorbance was measured with an autoplate reader at 492 nm, with 630 nm as the reference wavelength, on a microplate reader. The concentration of CAAF1 in the samples was obtained by interpolation of their absorbance from the calibration curve.

For immunohistochemical study, serial 3-5 mm thick sections were prepared from normal bovine fetal and newborn organs fixed with 4.5% formaldehyde in PBS, pH 7.4, and embedded in paraffin. Deparaffinized sections were stained by the streptavidin-biotin (SAB) method using a Histofine SAB kit (Nichirei Corp., Tokyo). All sections were treated with 0.3% H_2O_2 in methanol for 20 minutes for blocking the peroxidase activity of neutrophils. Purified IgG (1 μg/ml), GFD/CAAF1/22-5 was used for the primary antibody against CAAF1. Antibody binding was visualized by the peroxidase substrate, diaminobentidine.

RNA blotting and hybridization

Total RNA was prepared by the acid guanidinium-phenol-chloroform method described before (Oikawa et al., 1994) and poly(A)$^+$ RNA was isolated by Oligotex(dT) (JSR/Nippon Roche, Tokyo). For northern blot analysis, fifteen micrograms of total RNA was electrophoresed in 1% agarose gels and transferred onto Hybond-N nylon membranes (Amersham). The filters were prehybridized and hybridized at 42°C in 50% formamide, 5× SSC, 50 mM Na phosphate, pH 7.0, 0.5% SDS and 0.25 mg of denatured salmon sperm DNA/ml. DNA probes were labeled with a random primer labeling kit (Amersham). Filters were exposed to autoradiographic film with an intensifying screen.

RESULTS

Isolation of calcium-binding proteins from amniotic fluid

A low molecular mass with Ca^{2+} binding activity was detected in bovine amniotic fluid by the $^{45}Ca^{2+}$ overlay technique. As shown in Fig. 1, the band showed an apparent molecular mass of 8 kDa on Tricine/SDS-PAGE gels. In either the presence or the absence of β-mercaptoethanol, the activity migrated to the same position using Tricine/SDS-PAGE.

Ion exchange chromatography was used for the initial step of the purification of the Ca^{2+}-binding activity in bovine amniotic fluid. At this step, the activity was found to consist of two components showing apparent molecular masses of 9 kDa and 8 kDa, which were eluted from an S-Sepharose Fast Flow column at 30% 1 M CH_3COONH_4 as the first group and at 60-80% 1 M CH_3COONH_4 as the second group (Fig. 2). The fractions containing these two groups were separately collected and subjected to further purification steps. The 9 kDa Ca^{2+}-binding activity could be separated into two molecular masses by C_{18} reverse phase HPLC at the final step. The two proteins were colored yellow by silver staining, and the amino acid sequence analysis indicated that they were highly homologous, and they were considered to be isoproteins or the same protein (date not shown). The 8 kDa Ca^{2+}-binding activity was composed of a single molecule colored black by silver staining. Therefore we obtained at least two distinct CaBPs from bovine amniotic fluid, which showed apparent molecular masses of 9 kDa and 8 kDa. These migration patterns on Tricine/SDS-PAGE gels were distinct from that of S100α or S100β which showed an apparent molecular mass of 7 kDa (Fig. 3). Anti-S100α or S100β antibodies did not recognize the CaBPs in immunoblotting.

Since the yield of the 8 kDa protein was higher than that of

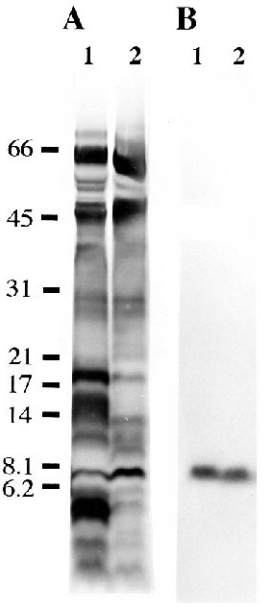

Fig. 1. Detection of Ca^{2+}-binding proteins in bovine amniotic fluid. (A) Tricine/SDS-PAGE analysis for proteins in amniotic fluid, silver staining. (B) ^{45}Ca autoradiograph of the blot of the same sample. Samples were treated with β-mercaptoethanol (lane 1) or not (lane 2). Size standards are in kDa.

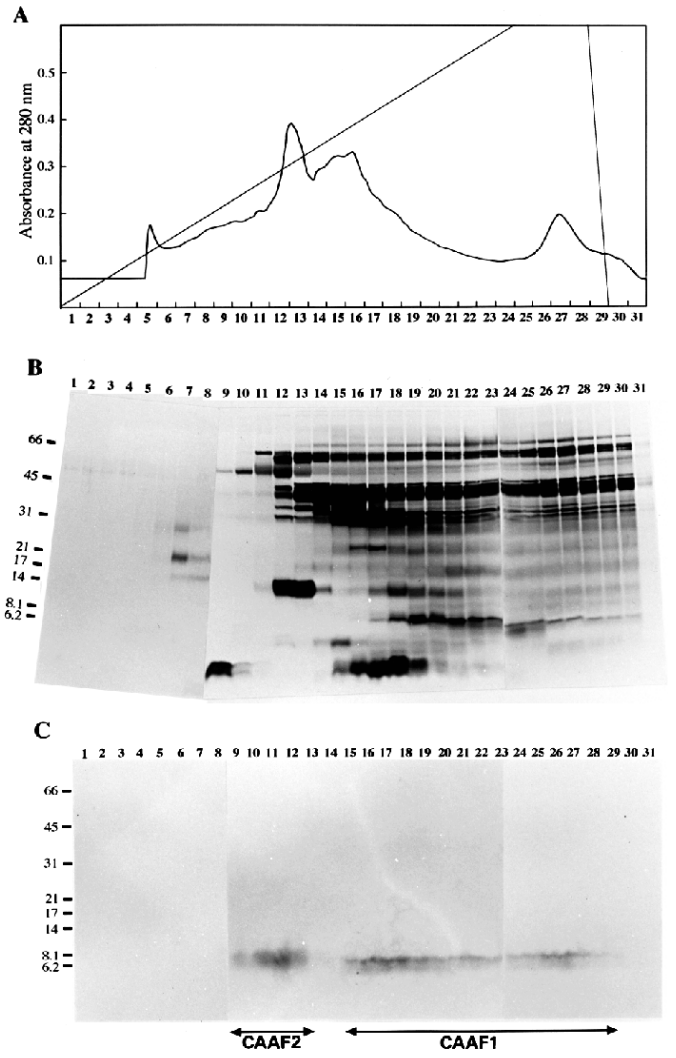

Fig. 2. Purification of Ca^{2+}-binding proteins in bovine amniotic fluid. (A) S-Sepharose Fast Flow ion exchange column chromatography of amniotic fluid. (B) Tricine/SDS-PAGE analysis of the separated fractions of S-Sepharose ion exchange column chromatography, silver staining. (C) ^{45}Ca autoradiograph of the blot of these separated fractions. Two Ca^{2+}-binding proteins, CAAF1 and CAAF2, are separated at this step. Size standards are in kDa.

the 9 kDa protein from bovine amniotic fluid, the 8 kDa CaBP was termed CAAF1 (calcium-binding protein in amniotic fluid 1) and the 9 kDa protein CAAF2. We attempted to determine the characteristics of CAAF1 in this study.

Amino acid sequence of CAAF1

The sequencing of intact CAAF1 determined the 51 amino acids of the N terminus of the protein from T_1 to P_{51}. Furthermore, the three fragments from the HPLC separation of lysylendopeptidase digestion, L1 (IFQDLDAD), L2 (DGAVSFEEFVVL) and L3 (TAHIDIHKE) and the two fragments from the HPLC separation of endopeptidase Glu-C digestion, V1 (LPKTLQNTKDQPTIDKIFQDLDADKDGAVSF) and V2 (EFVVLVSRVLKTAHIDIHKE) could be sequenced. The result of the sequence analysis of CAAF1 is shown in Fig. 4. The N-terminal 91 amino acid sequence was determined. All

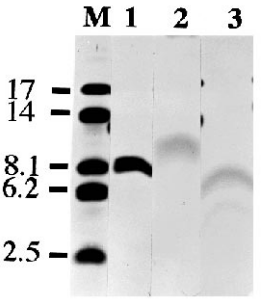

Fig. 3. Silver staining of purified Ca^{2+}-binding proteins in amniotic fluid (lane 1, CAAF1; lane 2, CAAF2) and bovine S100α (lane 3). Size standards are in kDa.

the sequences of the digested peptides were included in the 91 amino acid sequence. A computer homology search by FASTA and TBLASTX programs revealed CAAF1 to be a newly identified protein.

Nucleotide sequence of CAAF1

We obtained an authentic 263 bp cDNA probe for CAAF1 mRNA by the PCR technique using degenerate primers. The translated amino acid sequence of the probe was identical to that of codons 3 to 90 of CAAF1. With this probe, 10 positive clones

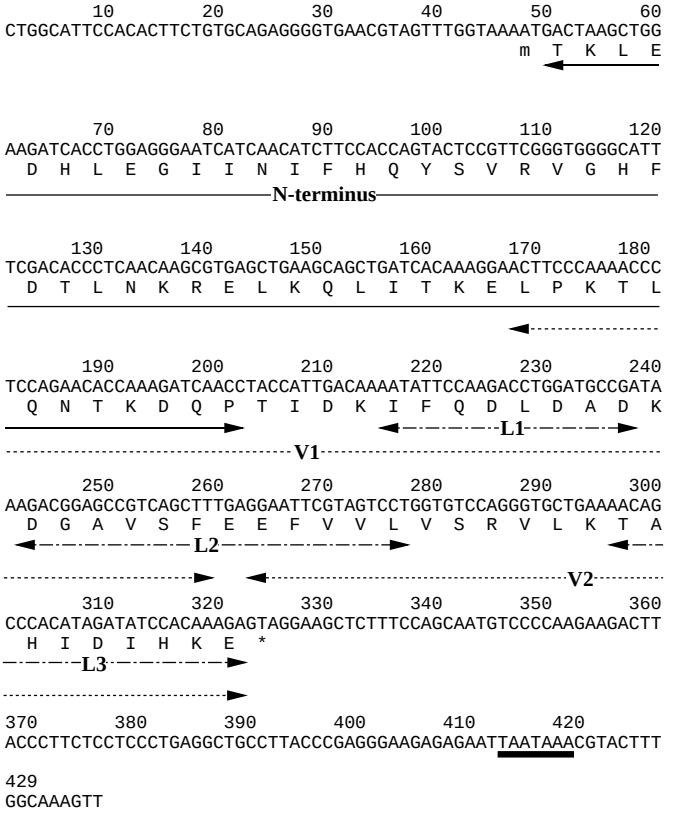

Fig. 4. Primary structure of CAAF1. The amino acid sequence of CAAF1 purified from bovine amniotic fluid is available from JIPID under accession number JP0080. The peptide fragments from CAAF1 are denoted according to their isolation by either lysyl endopeptidase cleavage (L) or endopeptidase Glu-C (V). The authentic CAAF1 consists of 91 amino acids without the initial methionine residue (m). The nucleotide sequence of CAAF1 cDNA is available from EMBL/GenBank/DDBJ under accession number D49548. The stop codon is indicated with an asterisk. The potential polyadenylation site is underlined.

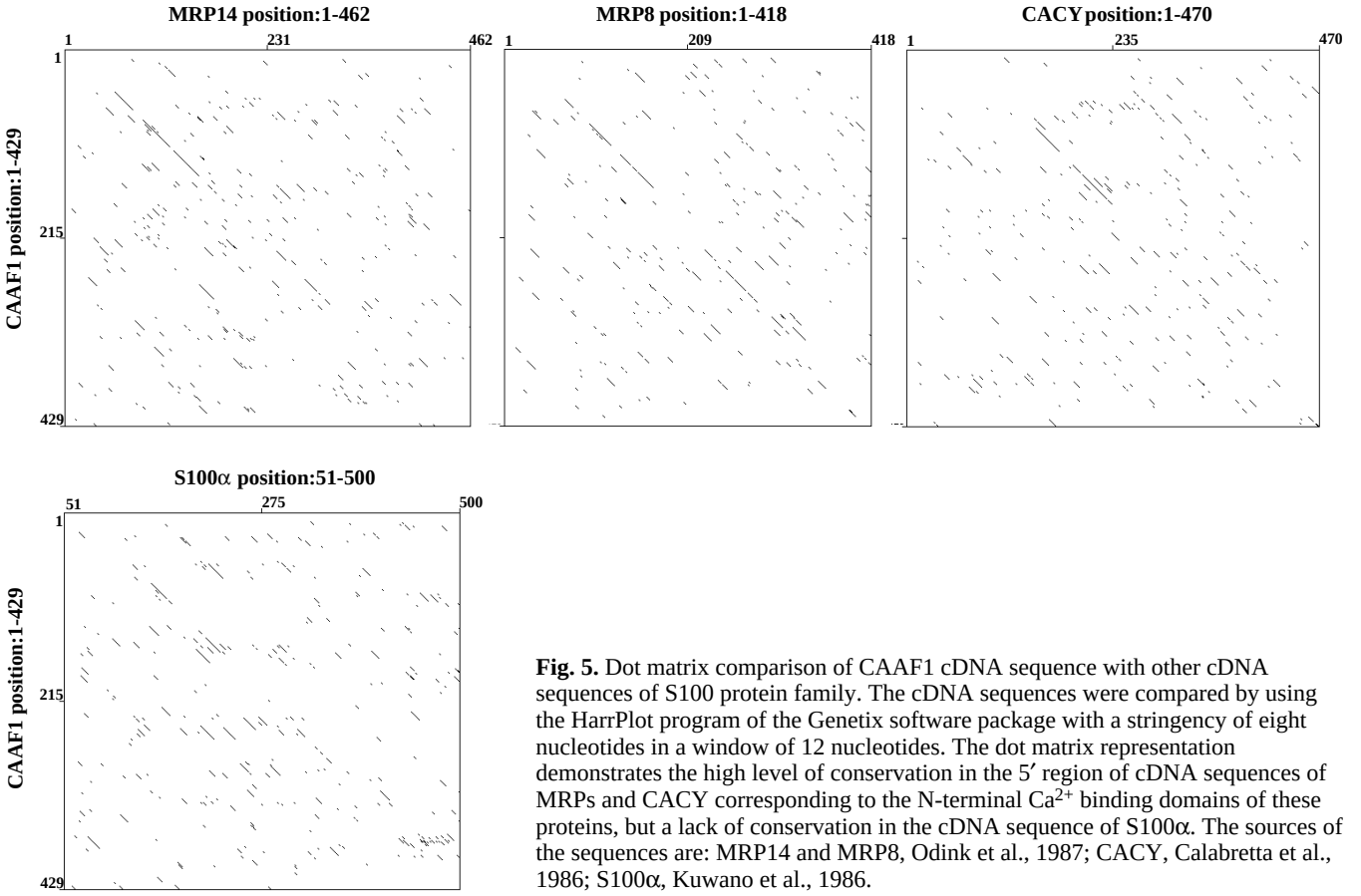

Fig. 5. Dot matrix comparison of CAAF1 cDNA sequence with other cDNA sequences of S100 protein family. The cDNA sequences were compared by using the HarrPlot program of the Genetix software package with a stringency of eight nucleotides in a window of 12 nucleotides. The dot matrix representation demonstrates the high level of conservation in the 5′ region of cDNA sequences of MRPs and CACY corresponding to the N-terminal Ca^{2+} binding domains of these proteins, but a lack of conservation in the cDNA sequence of S100α. The sources of the sequences are: MRP14 and MRP8, Odink et al., 1987; CACY, Calabretta et al., 1986; S100α, Kuwano et al., 1986.

A

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B.CAAF1      TKLEDHLEGIINIFHQYSVRVGHFDTLNKRELKQLITKELPKTLQNTKDQPTIDKIFQDLDADKDGAVSFEEFVVLVSRVLKTAHIDIHKE
P.CAGC .....................L..Y...I.............N..K.....G.......N...NQ.EQ...K......TD..I...DN....
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B

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B.CAAF1     -TKLEDHLEGIINIFHQYSVRVGHFDTLNKRELKQLITKELPKTL-QNTK-DQPTIDKIFQDLDADKDGAVSFEEFVVLVSRVLKTAHIDIHKE----------------------------
BEE22       MSQM.SSI.T...........L..Y...IQK.F...VQ....NF.KKQK.-NEAA.NE.ME...TNV.KQL.....IM..A.LTVAS.EEM.NTAPPGQGHRHGPGYGKGGSGSCSGQGSPDQ
B.S100a     GSE..TAM.TL..V..AH.GKE.DKYK.S.K...E.LQT..SGF.-DAQ.-.ADAV..VMKE..E.G..E.D.Q.Y....AALTVACNNFFWENS----------------------------
B.S100b     -SE..KAVVAL.DV.....G.E.DKHK.K.S...E..NN..SHF.-EEI.-E.EVV..VMET..S.G..ECD.Q..MAF.AMITTAC.EFF-EHE----------------------------
B.ICABP     -MSAKKSP.ELKG..EK.AAKE.DPNQ.S.E...L.LQT.F.SL.-----.-GPS.L.EL.EE..KNG..E......Q...KKISQ----------------------------------
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N-terminal homologous region — C-terminal homologous region

C

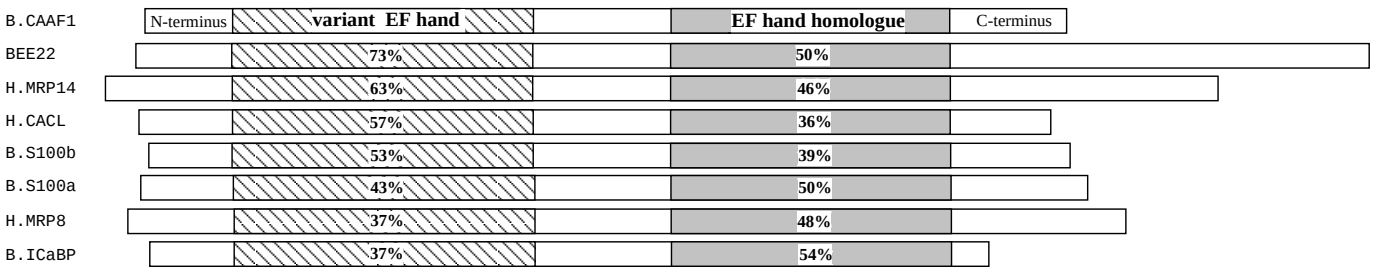


Fig. 6. Comparison of the amino acid sequences of CAAF1 and members of the S100 protein family. (A) Alignment of CAAF1 and CAGC amino acid sequences. Identical amino acid residues are represented by dots; vertical bars indicate gaps introduced for optimal alignment. (B) CAAF1 and S100 protein family. The amino acid sequences of BEE22, bovine S100α, bovine S100β, and bovine ICaBP aligned with CAAF1. The N-terminal and C-terminal homologous regions of these proteins are underlined, and are identical to the variant EF hand and the calmodulin EF hand homologue, respectively. (C) Schematic comparison of CAAF1 and S100 protein family. Closed boxes indicate the position of the variant EF hand and the calmodulin EF hand homologue. Sequence homologies were scored by taking into account the alignment of identical amino acids, and the percentage homology in the boxed regions is indicated.

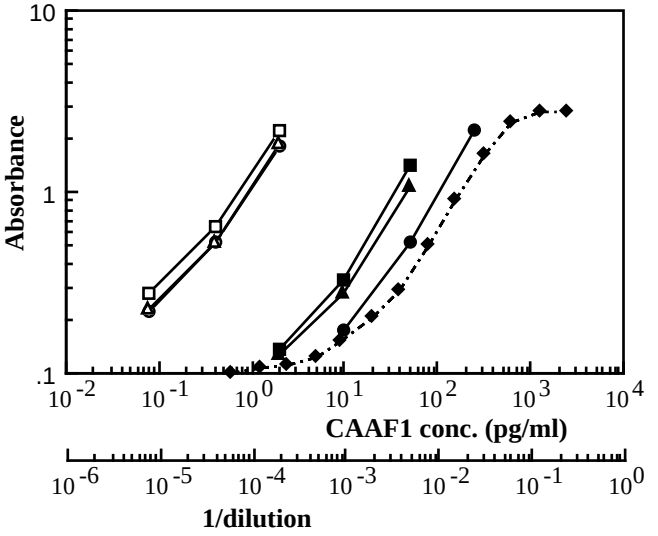
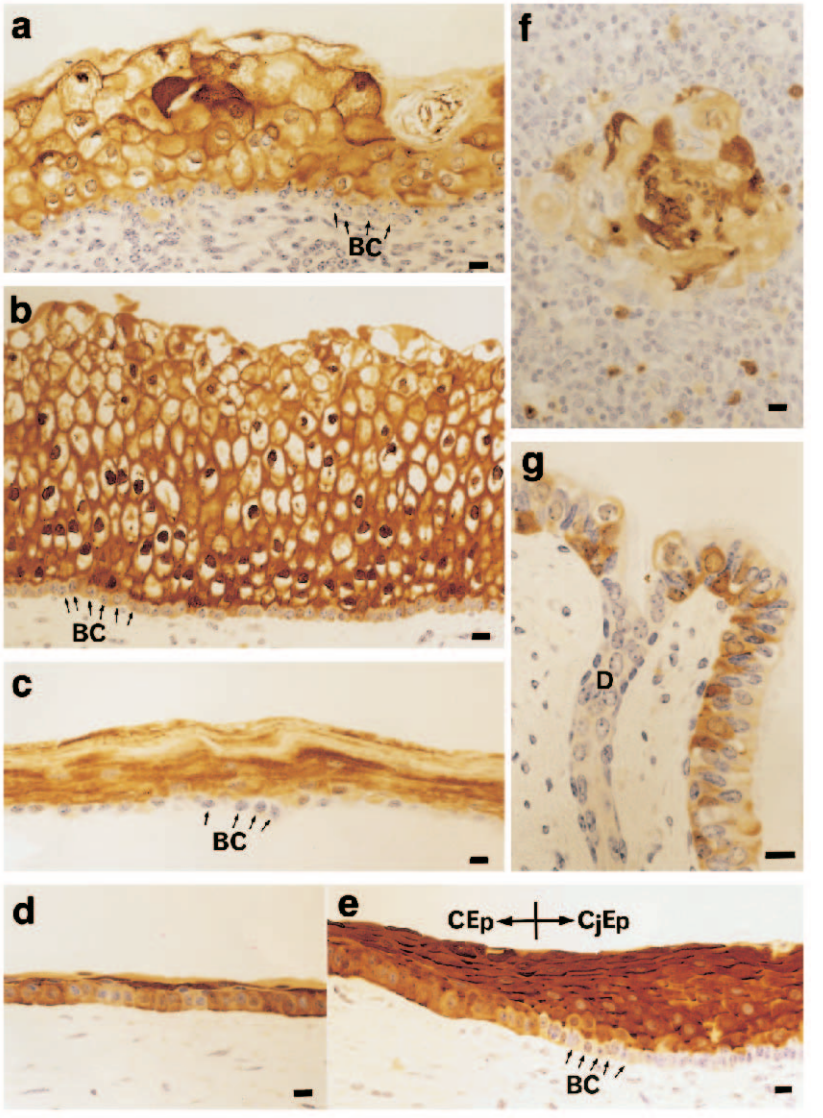


Fig. 7. Purified CAAF1, amniotic fluid and serum dilution curves. Purified CAAF1 (◆) at concentrations indicated, bovine amniotic fluid (□, △, ○) and three fetal sera (■, ▲, ●) were serially diluted and subjected to sandwich ELISA. Amniotic fluid and serum dilution is indicated as 1/dilution (fold^{-1}).

were obtained from a bovine fetal esophagus λgt11 cDNA library, and three independent clones λCAAF1/32, λCAAF1/34 and λCAAF1/51, were selected and further analyzed.

Fig. 4 shows the sequence of cDNA for CAAF1 mRNA, which contained 429 nucleotides. The sequence was confirmed for the three independent cDNA clones λCAAF1/32, λCAAF1/34 and λCAAF1/51. There was an open reading frame starting at nucleotide position 48 and ending with a termination codon at 324, which encoded a protein of 92 amino acids with a molecular mass of 10,685 Da. The deduced amino acid sequence of CAAF1 was completely identical to the sequence from the amino acid analysis of CAAF1 protein, with one exception: the initial methionine. CAAF1 protein has no cysteine residue, which allows the amino acids to form cova-



lently homo- or heterodimers. CAAF1 has no signal or membrane anchor sequences and lacks a consensus sequence for N-linked glycosylation. The polyadenylation signal sequence (AATAAA) is shown at nucleotide positions 407-412.

Comparison of amino acid sequences

Computer-aided homology analysis revealed that the cDNA sequence of CAAF1 is highly homologous with those of MRP8/CAGA (human), MRP14/CAGB (human) and calcyclin (CACY) (human), which are members of the S100 protein family; the extent of homology is 56.9%, 55.5% and 53.4%, respectively (Fig. 5).

At the protein level, CAAF1 also shares extensive homology with the S100 protein family of CaBPs, including S100α (bovine), S100β (bovine), S100L (bovine CaN19), vitamin D-dependent calcium-binding protein from bovine intestine (ICaBP), CACY (human), MRP14/CAGB (human), BEE22 (bovine MRP14/CAGB) and MRP8/CAGA (human), as the identities of 42.3%, 40.0%, 34.6%, 42.5%, 39.5%, 44.4%, 48.4% and 37.4%, respectively. During the preparation of this manuscript, a novel CaBP, calgranulin C (CAGC), was reported by Dell'Angelica et al. (1994). The CAGC was very abundant in pig granulocytes, and was purified from them. The amino acid sequence was 81% homologous with that of CAAF1 (Fig. 6).

Monoclonal antibodies to CAAF1

Five hybridoma clones, GFD/CAAF1/16-5, 20-5, 22-5, 25-25 and 31-5, were selected, for which the mAbs had the highest reactivity, not only with ^{125}I labeled CAAF1 in RIA, but also with purified natural CAAF1 in immunoblot analysis. The type of immunoglobulin of GFD/CAAF1/16-5, 20-5, 22-5, and 31-5 was IgG1, and that of GFD/CAAF1/25-25 was IgG2. The supernatants from these hybridomas specifically recognized the purified CAAF1 and the 8 kDa protein in the amniotic fluid, but the antibodies could not bind the bovine S100α or S100β. These mAbs were useful for the immunostaining of sections from formalin-fixed, wax-embedded tissues, except for the mAb of GFD/CAAF1/25-25. To determine whether the four mAbs (GFD/CAAF1/16-5, 22-5, 25-25, 31-5) bound the same or different epitopes on CAAF1, a competitive binding ELISA was performed to determine the ability of each biotinylated

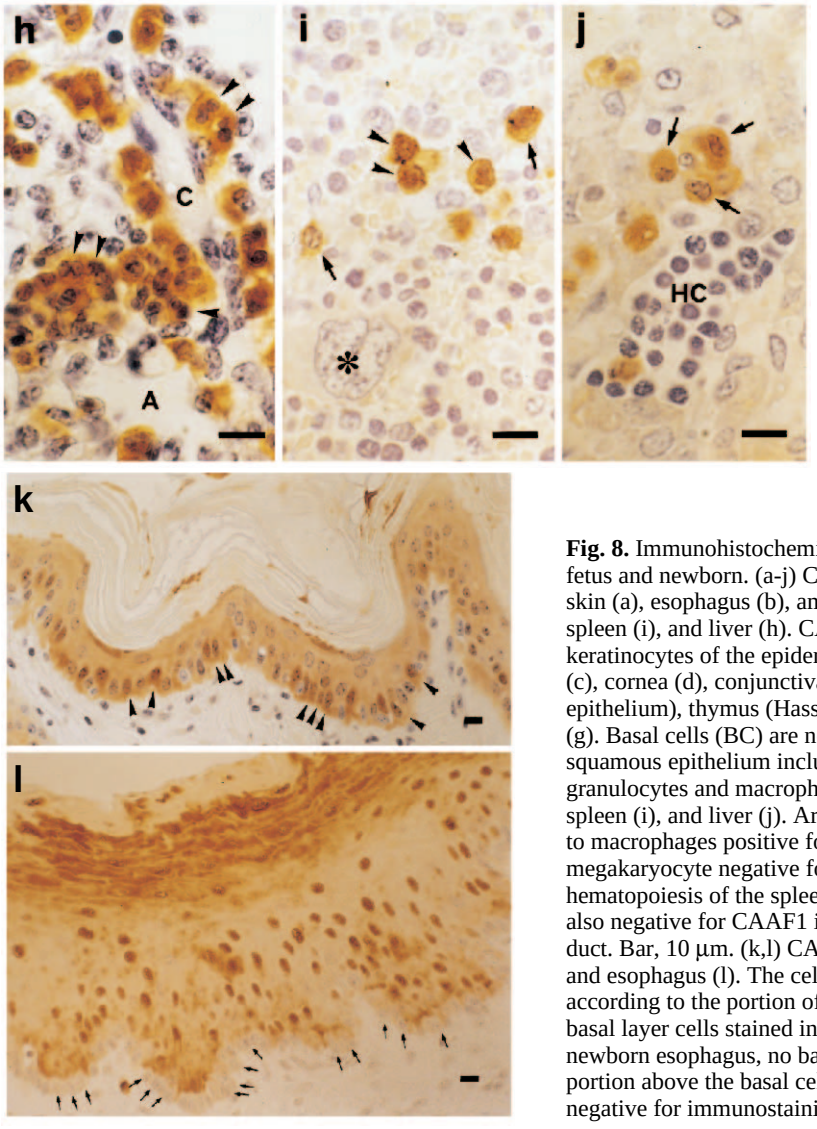


Fig. 8. Immunohistochemical detection of CAAF1 in various tissues from bovine fetus and newborn. (a-j) CAAF1 immunoreactivity in various bovine fetal tissues: skin (a), esophagus (b), amnion (c), eye (d and e), thymus (f), trachea (g), lung (h), spleen (i), and liver (h). CAAF1 immunoreactivity was strongly detected in keratinocytes of the epidermis (a) and epithelial cells of the esophagus (b), amnion (c), cornea (d), conjunctiva (e) (CEp, corneal epithelium; CjEp, conjunctival epithelium), thymus (Hassall's bodies) (f), and trachea (non-ciliated epithelial cells) (g). Basal cells (BC) are negative for CAAF1 immunoreactivity in the stratified squamous epithelium including the epidermis (arrows in a,b,c,e). Infiltrating granulocytes and macrophages also show CAAF1 immunoreactivity in the lung (h), spleen (i), and liver (j). Arrowheads indicate positive granulocytes, and arrows point to macrophages positive for immunostaining. The asterisk indicates a megakaryocyte negative for CAAF1 immunoreactivity in the extramedullary hematopoiesis of the spleen (e). Immature hematopoietic cells in the liver (HC) are also negative for CAAF1 immunoreactivity (j). A, alveolar space; C, capillary; D, duct. Bar, 10 μm. (k,l) CAAF1 immunoreactivity in bovine newborn epidermis (k) and esophagus (l). The cellular distribution of CAAF1 immunoreactivity changed according to the portion of the epithelium of the newborn. Arrowheads indicate basal layer cells stained in the nuclei in the epidermis (k). In the epithelium of newborn esophagus, no basal layer cells were positive, but the cells in the lower portion above the basal cells were stained in the nuclei. Arrows point to basal cells negative for immunostaining (l). Bar, 10 μm.

mAbs to bind to purified CAAF1 in the presence of a 100-fold excess of unlabeled mAbs. The unlabeled mAbs of GFD/CAAF1/16-5, 22-5, and 31-5 strongly inhibited all of these biotinylated anti-CAAF1 mAbs from binding to CAAF1. On the other hand, unlabeled mAb 25-25 did not prevent the biotinylated mAbs 16-5, 22-5, and 31-5 from binding to CAAF1. These results suggest that mAbs 16-5, 22-5, and 31-5 recognize the same epitope or a closely related binding site on CAAF1, and that the epitope of mAb 25-25 is far enough away from the other recognition sites to cause no steric hindrance.

ELISA for CAAF1 in amniotic fluid

According to the results of the competition assay of mAbs, we selected mAb 22-5 for the immobilized antibody and mAb 25-25 for the labeled antibody, and developed a sandwich ELISA system. Fig. 7 shows the ELISA calibration curve obtained by using known amounts of purified CAAF1. The minimum detectable concentration was about 10 pg/ml. The ELISA study showed that immunoreactive CAAF1 was present at concentrations of 1.2, 1.2, and 1.5 μg/ml (n=3) in amniotic fluid, and 17, 40, and 44 ng/ml (n=3) in fetal serum. On other hand, adult sera contained the immunoreactive CAAF1 at concentrations of 56 and 87 ng/ml (n=2). The dilution curves of the amniotic fluid samples and serum samples were parallel to those using purified CAAF1 as calibrator (Fig. 7), indicating that the structure of the active materials present in these samples was immunologically indistinguishable from that of purified CAAF1.

Tissue and cellular distribution of CAAF1

To reveal the cellular distribution of CAAF1, a wide variety of normal tissue samples were examined with the purified mAb of GFD/CAAF1/22-5 by using the SAB staining method.

Table 1 summarizes our findings with the fetal tissues studied. The normal fetal tissues found to be antigen-positive were the epidermis of the skin, the stratified squamous epithelium of the esophagus, the epithelia of the cornea and conjunctiva, Hassall's bodies in the thymus and the bronchial epithelium. Of the extrafetal organs, the amnion from the umbilical cord and the placenta were also positive. The positive cells were the squamous epithelial cells from the epidermis, esophagus, cornea, conjunctiva and amnion, and non-ciliated epithelial cells of the bronchial epithelium (Fig. 8a-g).

In the epidermis and the esophageal epithelia of the fetus, basal layer cells were negative with anti-CAAF1 antibodies and the positively stained cells abruptly appeared in the second layer just above the basal cells. All cells throughout the upper layer, in which the cells were undergoing terminal differentiation, reacted strongly with the antibodies. No stained cells were observed in sweat ducts or hair follicles in the epidermis.

We also examined newborn epidermis and esophageal epithelium in addition to fetal organs (Fig. 8k,l). The epidermis and esophageal epithelia were also positive, but the cellular distribution of the antigen changed according to the portion of the epithelium of the newborn. In the epidermis of newborn skin, some cells in the basal layer were stained strongly in the nuclei, and the cells in the middle portion were stained predominantly in the cytoplasm, and the nuclei were becoming discolored. The cells of the keratin layer without nuclei from the epidermis were negative. On the other hand, hair follicle cells and sweat duct cells were also negative. In the epithelia of newborn esophagus, no basal layer cells were positive, but the cells in the lower portion above the basal cells were stained strongly in the nuclei, and the cells in the middle portion were stained predominantly in the cytoplasm. The cells of the keratin layer without nuclei were also negative.

All the organs, especially lung, spleen, and liver, contained many positive hematopoietic cells which were identical to polymorphonuclear cells and macrophages. In the spleen and liver, extramedullary hematopoiesis was observed, but their immature cells were negative for CAAF1. Megakaryocytes were also negative. Only mature cells having pleomorphic nuclei were stained positive: they were considered to be neutrophils and some macrophages. These positive cells were

Table 1. Tissue and cellular distribution of CAAF1 in bovine fetus

Organ	IR	Tissue/Cell
Central nervous system		
Cerebrum	–	
Cerebellum	–	
Brain stem	–	
Spinal cord	–	
Cardiovascular system		
Great artery	–	
Great vein	–	
Heart	–	
Lymphoid tissue		
Thymus	+	Hassall's body
Spleen	+	PMN, macrophage
Digestive system		
Parotid gland	–	
Submandibular gland	–	
Esophagus	+	Epithelium/squamous cell
Stomach	–	
Duodenum	–	
Jejunum	–	
Colon	–	
Rectum	–	
Liver	–	
Pancreas	–	
Respiratory system		
Trachea	+	Epithelium/non-ciliated epithelial cell
Lung	+	Epithelium/non-ciliated bronchial epithelial cell
Urinary system		
Kidney	–	
Ureter	–	
Bladder	–	
Sexual organ		
Testis	–	
Endocrine organs		
Pituitary gland	–	
Adrenal gland	–	
Skin		
Skin	+	Epidermis/keratinocyte
Sense organ		
Eye	+	Epithelium/squamous cell
Lachrymal gland	–	
Others		
Hematopoietic cells	+	PMN, macrophage
Placenta	+	Amnion/squamous cell
Umbilical cord	+	Amnion/squamous cell

IR, immunoreactivity; PMN, polymorphonuclear leukocyte.

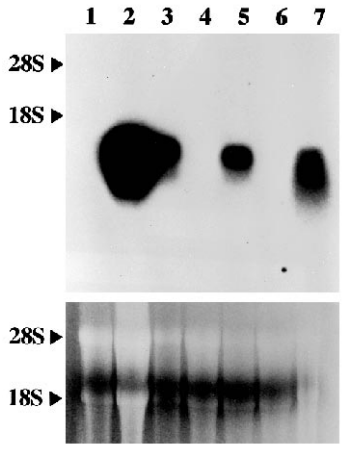


Fig. 9. Northern blot analysis of CAAF1 mRNA expression in various tissues: cerebrum (lane 1); esophagus (lane 2); lung (lane 3); liver (lane 4); spleen (lane 5); small intestine (lane 6); and skin (lane 7). The blots were hybridized with CAAF1 cDNA probe. Hybridization of CAAF1 cDNA shows a 0.9 kb mRNA. Lower panel, ethidium bromide staining of the RNA gel prior to transfer. The amount of RNA in each lane was monitored using 28 S and 18 S rRNA.

stained not only in the cytoplasm but also in the nuclei. No lymphocytes in the organs were positively stained (Fig. 8h-j).

Northern blot analysis of CAAF1 in bovine fetus

To confirm the results of the immunohistochemical study, we examined fetal tissues for the expression of specific mRNA for CAAF1 by northern blot analysis.

As shown in Fig. 9, a single band was detected at about 0.9 kb in the organs examined. CAAF1 mRNA was most abundant in the esophagus, and intermediate levels were found in the skin, lung and spleen, but not in the brain.

DISCUSSION

In this study, we found a novel CaBP, CAAF1, in bovine amniotic fluid by using a Ca^{2+} overlay technique, isolated this molecule, and determined the amino acid sequence and nucleotide sequence of the mRNA for the molecule. We also produced monoclonal antibodies against CAAF1, and revealed its tissue and cellular distribution by ELISA, immunohistochemistry, and northern blot analysis.

Primary structure of CAAF1

We identified at least two different molecules of CaBPs in bovine amniotic fluid, termed CAAF1 and CAAF2, with apparent molecular masses of 8 kDa and 9 kDa on Tricine/SDS-PAGE gels, respectively. Since the yield of CAAF1 from bovine amniotic fluid was greater than that of CAAF2, we determined the characteristics of CAAF1.

CAAF1 amino acid sequence analysis revealed that authentic CAAF1 is a protein with a molecular mass of 10,554 Da, consisting of 91 amino acids. This was confirmed by cDNA sequence analysis. The amino acid sequence was completely identical to the deduced amino acid sequence from the cDNA of CAAF1, except for the initial methionine residue. CAAF1 has no cysteine residue, indicating that CAAF1 dimerization through interchain disulfide bonds is impossible. CAAF1 also lacks both the predicted signal peptide sequence and consensus sequence of N-linked glycosylation. Therefore it is possible to postulate that CAAF1 differs from general secretory proteins, but that two hydrophobic domains are present in both the N terminus and the C terminus of CAAF1, which may function as internal signal sequences.

A computer search revealed that CAAF1 is homologous to the S100 protein family of small CaBPs. The amino acid sequence of CAAF1 was 70% homologous to calgranulin C (CAGC), which is the most closely related protein (Fig. 6A). CAGC is a novel member of the S100 protein family and is very abundant in the porcine granulocyte (Dell'Angelica et al., 1994). According to a comparison of the amino acid sequence of CAAF1 and other members of the S100 protein family, including BEE22 (bovine MRP14) (Tang et al., 1993), bovine ICaBP (Fullmer and Wasserman, 1981), bovine S100α (Isobe and Okuyama, 1981), and bovine S100β (Jensen et al., 1985) (Fig. 6B), they have an identical characteristic configuration in the N-terminal region and the C-terminal region. In the N-terminal site corresponding to the 30 amino acids of CAAF1 (IINIFHQYSVRVGHFDTLNKRELKQLITKE), the homologies between CAAF1 and other proteins are 37 to 73%. At the C-terminal site corresponding to the 28 amino acids of CAAF1 (IDKIFQDLDADKDGAVSFEEFVVLVSRV), the homologies are 36 to 54%. In addition, the C-terminal site of CAAF1 is strongly homologous (38.5%) to the sequence of the forth Ca^{2+}-binding domain of calmodulin, which displays a typical helix-loop-helix conformation (EF hand) (Babu et al., 1985; Kretsinger, 1980), and this CAAF1 site also shows a helix-loop-helix conformation in Chou-Fasman's secondary structure prediction using Genetyx software. On the other hand, the N-terminal site of CAAF1 has no such homologous region in the sequence of calmodulin. The N-terminal homologous part of the S100 protein family is identical to the N-terminal EF hand structure of ICaBP, a variant EF hand, which differs from the canonical EF hand considered to be a putative Ca^{2+}-binding domain of calmodulin (Szebenyi et al., 1981; Szebenyi and Moffat, 1986). In this N-terminal motif, CAAF1 is especially homologous (73.3%) to BEE22, out of the members of the S100 protein family. The homology between CAAF1 and MRP14 is also high, 63.3% (Fig. 6C). In comparing the cDNA sequence of CAAF1 and MRP14 (Odink et al., 1987), we found that the highly homologous area corresponds to this N-terminal motif, variant EF hand, according to the dot matrix study, whereas there is no such homologous area in the cDNA sequence of bovine S100α (Kuwano et al., 1986) (Fig. 5).

These data indicate that CAAF1 must have at least one Ca^{2+}-binding site, of which the C-terminal EF hand conforms well to the known functional calmodulin sequence, and CAAF1 is a new member of the S100 protein family, structurally closely related to MRP/calgranulin proteins.

CAAF1 in squamous epithelium

To gain insight into the biological functions of CAAF1, we obtained five monoclonal antibodies from hybridoma clones, 16-5, 20-5, 22-5, 25-25 and 31-5. All these mAbs recognized CAAF1 from among proteins of the amniotic fluid in an immunoblot analysis, but they did not bind other members of the S100 protein family including purified bovine S100α,or S100β, or recombinant human MRP8 or MRP14 (J. H., unpublished observations). All of these mAbs except mAb GFD/CAAF1/25-25 were useful for immunostaining. To determine the cellular distribution of CAAF1, a battery of normal tissue samples was examined with mAb GFD/CAAF1/22-5. Similar staining was also obtained with the other three mAbs.

Our immunohistochemical studies and RNA blotting analysis revealed that CAAF1 was produced by the squamous epithelial cells including those of the amnion and hematopoietic cells, which were identified as neutrophils and some macrophages throughout the fetal body. In the epidermis or mucosal stratified squamous epithelia of the fetus, no basal layer cells were stained with anti-CAAF1 antibodies, while both the cytoplasm and nuclei of the upper layer cells were strongly stained. The upper layer cells showed a tendency toward squamous cell differentiation, if not morphologically complete. In the myeloid lineage, mature cells with pleomorphic nuclei were stained positively. These findings taken together indicated that the fetal immature proliferating cells probably do not produce CAAF1.

On the other hand, in the newborn, the squamous epithelial cells showed terminal differentiation in the epidermis and esophagus. The cellular distribution of CAAF1 in these stratified squamous epithelial cells depended on the stage of cell differentiation. The nuclei were positive in the immature cells located just above the basal layer cells, and the cytoplasm became positive in differentiated cells in the upper layer. In addition, the terminal differentiated cells in the keratin layer were not stained by CAAF1 antibodies.

These findings strongly suggest that CAAF1 is one of the stage-specific proteins in the differentiation of squamous epithelial cells. The difference in distribution of CAAF1 between fetal and newborn squamous epithelium indicates that cells expressing CAAF1 in newborns have certain embryonic characteristics in the squamous epithelium.

CAAF1 in amniotic fluid

The results of the competitive binding ELISA suggested that mAb GFD/CAAF1/25-25 and other mAbs recognized the distinct epitopes of CAAF1. On the basis of the combination of two mAbs which have different epitopes, we developed a sensitive sandwich assay system for CAAF1 with mAb 22-5 and biotinylated mAb 25-25.

This ELISA system revealed that CAAF1 was present in amniotic fluid at a concentration of about 120 nM, and about 4 nM in the fetal serum and 7 nM in the adult serum. There are some reports that CaBPs in body fluids, S100β and MRP/calgranulin proteins have some biological activity (Kligman and Marshak, 1985; Murao et al., 1990). But there are no reports about CaBPs in amniotic fluid. The amount of CAAF1 in amniotic fluid is very large, when compared with extracellular concentrations of S100β or MRP/calgranulin proteins (Shashoua et al., 1984; Brüggen and Cerletti, 1991).

The concentration of CAAF1 in amniotic fluid is over 30 times as high as that in fetal serum; therefore it is possible to speculate that CAAF1 in amniotic fluid comes from the epithelial cells covering the fetus, not from hematopoietic granulocytes. These data indicate that CAAF1 is preferentially produced by fetal squamous epithelial cells including epidermal keratinocytes and amniotic epithelial cells, and that it might be stored in the amniotic fluid via an unknown pathway from the squamous epithelial cells during embryogenesis.

CAAF1 and MRP/calgranulins in the S100 protein family

MRP/calgranulin proteins are produced by not only granulocytes and macrophages, but also squamous epithelial cells (Wilkinson et al., 1988; Tang et al., 1993). Our findings indicate that CAAF1 and MRP/calgranulin proteins have a distinct but overlapping distribution in those cells. CAAF1 and MRP proteins might share biological as well as structural characteristics. Each member of the S100 protein family exhibited a quite different expression pattern in mammalian tissues (Kligman and Hilt, 1988). But the similarities between CAAF1 and MRP/calgranulin proteins in both structure and cell type-specificity of the expression are especially unique among the S100 protein family, and it might be proposed that CAAF1 and MRP/calgranulin proteins constitute a subfamily of the S100 protein family. However, there are no comparative studies of the biological features of CAAF1 and MRP/calgranulins; it is therefore necessary to examine thoroughly the expression of CAAF1 and MRP/calgranulins in squamous epithelium for understanding their biological functions.

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Note added in proof

Recently the N-terminal sequence of human p6 neutrophil protein was published by Guignard et al. (1995). Identification and characterization of a novel human neutrophil protein related to the S100 family. *Biochem J.* **309**, 395-401. Only the first 20 amino acid residues are available but these are identical to CAAF1 (except for two conservative changes) and porcine CAGC (except for three changes).