Identification and Cloning of a Secreted Protein Related to the Cysteine-Rich Domain of Frizzled
Evidence for a Role in Endothelial Cell Growth Control

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Abstract—We report the isolation of a cDNA, FrzA (frizzled in aorta; GenBank accession No. U85945), from bovine aortic endothelium. It is the bovine counterpart of the mouse sFRP1, which encodes for a secreted protein that is homologous to the cysteine-rich domain of frizzled. Members of the frizzled family of genes have been shown to be required for tissue polarity and to act as receptors for Wnt. The predicted protein product of this gene includes the cysteine-rich extracellular domain, but not the 7 putative transmembrane domains that are highly conserved among members of the frizzled family. Visualization of FrzA mRNA and protein revealed that it was widely distributed among adult tissues. FrzA is expressed by highly differentiated or polarized cells, eg, neurons, cardiocytes, or various epithelia. Analysis of its expression in endothelium revealed that FrzA mRNA levels were high in endothelial cells scraped from freshly obtained bovine aortas, decreased when cells were placed in culture and began to proliferate, but increased at confluence. Transient transfection assays and an assay using addition of purified protein indicate that FrzA reduces the proliferation of endothelial cells. These data demonstrate the existence of a secreted protein homologous to the extracellular domain of the fz receptor, which we speculate plays a role in controlling cell growth and differentiation, possibly by regulating accessibility to Wnt family members. (Circ Res. 1999;84:1433-1445.)

Key Words: cloning ■ bovine-secreted frizzled-related protein ■ tissue expression ■ endothelial differentiation

Increasing evidence indicates a role for cell-cell interactions in the regulation of cell growth and differentiation, as well as in the formation of organ and tissue structures. Genetic studies in Drosophila melanogaster have revealed that the frizzled (fz) locus participates in such intercellular communication. The product of the fz gene functions in Drosophila to transmit information regarding tissue polarity,1 organizing hair and bristle orientation throughout the cuticle. Genetic mosaic experiments have demonstrated fz function in the transduction of the polarity signal.2 A large family of putative transmembrane receptors homologous to fz has been described in mammals.3,4 These proteins are conserved from flies to mammals, which suggests functional parallels between Drosophila fz and its homologues or related genes in other species. Additional families of highly conserved genes are involved in the determination of cell and tissue polarity, including Notch5 and Wnt/wingless.6,7

All fz sequences share the same overall domain structure with a signal peptide, a unique and highly conserved cysteine-rich domain (CRD), and 7 putative transmembrane domains. Frizzled family members are expressed in diverse organisms, show specific tissue distribution, and are regulated during embryonic development. The role of fz proteins as transmembrane proteins with potential receptor function has attracted much attention.5 A recent in vitro study demonstrated that frizzled proteins could function as receptors for Wnt (Wingless).9,10 The Wnt family includes numerous genes isolated from diverse species, which encode secretory glycoproteins that appear to influence multiple biological responses, including cell growth, differentiation, and survival.6,11,12 The importance of the fz-Wnt interaction was highlighted by the fact that cells transfected with soluble Wg protein bind both Drosophila and mammalian fz proteins and initiate signal.9,10 Furthermore, and relevant to our observations, the extracellular CRD of fz was shown to be required for Wnt ligand binding.

We report the isolation and characterization of the bovine orthologue of mouse sFRP1, FrzA (frizzled in aorta; GenBank accession No. U85945), which encodes a deduced protein that contains a frizzled CRD, but no transmembrane domains. It belongs to a novel family of secreted mammalian proteins related to the frizzled family, sFRP, but encoding only for the CRD.9,10,13–16 We document that in adult tissue the transcripts and the protein were widely distributed but had specific cellular localization. The expression of FrzA in bovine aortic endothelial cells (BAECs) was examined, and FrzA mRNA was found to be expressed in cells in situ, diminished with increasing passage of the cultured cells, and
inducible by serum-free culture conditions. Moreover, results of transient transfection studies and an assay using exogenously added purified protein assays implicate FrzA in the control of endothelial and smooth muscle proliferation. These data indicate the existence of a secreted protein, a member of the secreted frizzled-related protein (sFRP) family consisting of a fz-like CRD, which we hypothesize plays a role in regulating cell growth and differentiation in adult organisms, perhaps by controlling accessibility to Wnt family members.

Materials and Methods

**cDNA Library Construction**

RNA was isolated by a CsCl gradient from endothelium scraped directly from bovine aortas. Poly(A)+ RNA was isolated by chromatography on an oligo(dT)-cellulose column (Stratagene). A cDNA library was constructed in the Uni-ZAP XR vector using the ZAP-cDNA synthesis kit (Stratagene) and size fractionated on a column following the manufacturer’s instructions. The yield was 500 000 primary recombinants with an average size of 2 kb.

Approximately 600 000 phages plaques were absorbed onto nylon membrane (New England Nuclear Research Products) and screened. Four successive hybridization rounds were carried out to obtain a complete cDNA sequence. The cDNA tag (A1), which was initially isolated from vessel endothelium by differential display, was used as a probe for the first round of hybridization. A probe, corresponding to 300 bp of the 5’ end of the clone obtained in the first round of screening, was used in the second screening, and the same strategy was applied in 3 subsequent screens. The probes were random-prime labeled (Rediprime, Amersham), and hybridization was carried out in a mixture containing 1% SDS, 2× SSC, 10% dextran sulfate, 50% formamide, 5× Denhardt’s solution, and 50 ng/ml ssDNA. The filters were washed successively in 2× SSC-0.1% SDS at room temperature for 20 minutes and then twice in 0.5× SSC-0.1% SDS at 50°C and were then exposed to x-ray film. Positive clones were isolated and rescreened until they were plaque purified. The inserts in AZAP vector were excised using the protocol recommended by the manufacturer (Stratagene). The structure of the double-stranded phagemid cDNA insert was determined by the cycle-sequencing method using fluorescent dideoxy terminator nucleotides with an Applied Biosystems 373A automated DNA sequencer (Dana-Farber Cancer Institute Core Facility); the sequence was determined for the ZAP vector using the Maxiscript kit (Ambion). Reactions were incubated for 1 hour at 37°C, and full-length radiolabeled probes were purified by electrophoresis through a denaturing polyacrylamide gel. Transcripts were located in the gel by autoradiography and eluted from gel slices. Reagents were obtained from Ambion, and the hybridization and digestion conditions were as recommended by the manufacturer.

**Protein and Nucleic Sequence Analysis**

Sequence analysis was carried out using the GCG package (Genetic Computing Group). Homology searches against a protein databank were accomplished with the Blast algorithm. Alignment of 2 sequences was accomplished with Bestfit and of multiple sequences with Pileup algorithms.

**Cell Culture**

BAECs were isolated as previously described by scraping the intimas of bovine aortas with a scalpel blade. Bovine smooth muscle cells (BSMCs) were explanted from bovine aortas as previously described and used at passages 2 to 10. All cells were grown in DMEM (Sigma) with 10% FCS (Hyclone Laboratories, Inc.), L-glutamine (29.2 mg/mL), and penicillin-streptomycin (10 000 U/mL) (Irvine Scientific) (DMEM/10% FCS). For each experiment, endothelial cells (ECs) scraped from several aortas were pooled and were either processed for RNA isolation or cultured in DMEM/10% FCS. Once cells had reached confluence, they were switched for 24 hours to medium with serum or serum-free medium containing BSA (1%), insulin (1 μg/mL), transferrin (200 μg/mL), ascorbate (0.2 mmol/L), and sodium selenite (6.25 ng/mL) (all from Sigma).

**Northern Blot Analysis**

Tissue obtained directly from the slaughterhouse was frozen immediately in liquid nitrogen. Total RNA was isolated by homogenizing the tissues in RNAzol B (Tel-Test, Inc) following the manufacturer’s instructions. Total RNA (20 μg) was electrophoresed in a 1% agarose/formaldehyde gel and transferred by capillary technique to a GeneScreen membrane (NEN Research Products). An EcoRI/SmaI fragment of FrzA cDNA was used as a probe, and hybridization was carried out with random-labeled probe.

**RNase Protection**

Total RNA was prepared from adult bovine brain, retina, spleen, heart, kidney, liver, muscle, and aorta (adventitia, media, and endothelium were extracted separately) in RNAzol and desalted using a GeneClean kit (BIO 101). Total RNA was extracted from cultured cells by a CsCl step gradient.

Ten micrograms of total RNA from the aortic adventitia, media, endothelia, and cultured BAECs or 50 μg of each of the other tissues (or tRNA as a control) was assayed by RNase protection. The cRNA probes for GAPDH and FrzA were prepared as follows. A GAPDH cDNA, amplified by polymerase chain reaction (PCR) using 5′-TGAAGGTCCCCAGTAACGGGA as sense primer and 5′-CATGTTGGCCATGAGTCCA as antisense primer, was cloned into the pCRRII using a T/A cloning kit (Invitrogen). The primer sequences were chosen on the basis of the human sequence (Clontech). The construct was digested with EcoRI and AccI, subcloned into the pBluescript II KS with or without plasmid in EcoRI/AccI sites, and transcribed. A riboprobe for FrzA was synthesized from the A1-tag PCR-amplified product cloned into the pcRI1000 plasmid (Invitrogen). Transcription was carried out with bacteriophage T3 RNA polymerase for 5′ end-linearized FrzA vector and GAPDH vector using the Maxicscript kit (Ambion). Reactions were incubated for 1 hour at 37°C, and full-length radiolabeled probes were purified by electrophoresis through a denaturing polyacrylamide gel. Transcripts were located in the gel by autoradiography and eluted from gel slices. Reagents were obtained from Ambion, and the hybridization and digestion conditions were as recommended by the manufacturer.

**In Situ Hybridization**

Adult bovine tissues obtained directly from the slaughterhouse were fixed in 4% formaldehyde in PBS for 24 hours and embedded in paraffin. In situ hybridization was carried out using the Genius Labeling and Detection Kit (Boehringer Mannheim), as described elsewhere. Serial 5-μm sections were fixed in 4% paraformaldehyde for 30 minutes, dehydrated, treated with proteinase K (20 μg/mL) for 15 minutes, and then acetylated with triethanolamine, dehydrated, and air dried.

Indicated single-stranded RNA probes were prepared using digoxigenin-11-UTP according to the manufacturer’s instructions. For a FrzA probe, a 2-kb cDNA fragment containing nearly the entire 3′-untranslated region of FrzA cDNA was used. The cDNA, subcloned into the polylinker region of pBluescript KS, was linearized by digestion with the appropriate enzymes to allow synthesis of a digoxigenin-labeled RNA in either the sense or the antisense orientation (using T3 or T7 RNA polymerase, respectively). To obtain small RNA fragments of ~100 bp, the RNA transcript was partially hydrolyzed in 60 mmol/L Na2CO3/40 mmol/L NaHCO3 at 60°C for 1 hour and ethanol precipitated. Hybridization was carried out at 50°C for 16 hours with 200 ng/mL of probe in a hybridization buffer containing 50% deionized formamide, 1× Denhardt’s solution, 4× SSC, 10% dextran sulfate, and 0.4 mg/mL ssDNA. Sections were washed twice for 15 minutes in 2× SSC at room temperature, treated with RNase A (100 μg/mL) in 2× SSC at 37°C for 30 minutes, and then washed twice for 20 minutes in 2× SSC at 42°C and then at room temperature. The alkaline phosphatase–conjugated anti-digoxigenin antibody (1:500 dilution) was incubated for 2 hours in 10% horse serum at room temperature, and the sections were rinsed overnight. The sections were incubated for 8 hours with the substrate nitroblue tetrazolium salt X-phosphate and then counterstained with the methyl green nuclear stain (Vector Laboratories).
Production of Polycyonal Antiserum and Immunohistochemistry Analysis

Oligopeptides corresponding to C-terminal amino acids 206 to 218 and 269 to 296 were synthesized by Research Genetics, Inc. The peptides were conjugated to keyhole-limpet hemocyanin, emulsified with an equal volume of Freund’s complete adjuvant, and used to immunize New Zealand White rabbits. Injections were repeated at 4-week intervals, and the sera were obtained 10 weeks after the initial injection.

Immunoperoxidase staining was performed on serially cut 3-μm sections. Endogenous peroxidase was neutralized with 3% hydrogen peroxide, and the sections were preincubated in blocking serum (10% goat serum in PBS) for 20 minutes at room temperature. The sections were incubated in primary antibody (1:5000) overnight at 4°C in PBS, 5% goat serum, 2% BSA, and 0.2% Tween and were then incubated with biotinylated rabbit secondary antibody (Amersham) (1:100) for 30 minutes at room temperature in PBS with 5% goat serum, 2% BSA, and 0.2% Tween. The sections were rinsed with PBS and visualized by incubation in 3,3′-diaminobenzidine tetrahydrochloride dihydrate (Vector Laboratories). Sections were counterstained with 10% Gill hematoxylin. For each tissue section labeled with FrzA antibody, a negative control was prepared by staining a serially cut section with a nonspecific rabbit polyclonal antibody.

Protein Cellular Distribution and Purification

COS-7 cells were stably transfected with the pcDNA3 mammalian expression vector (Invitrogen) containing FrzA fused in the C terminus with a myc::his epitope using an activated-deutrimer reagent (Superfect, Qiagen) according to the manufacturer’s protocol. Stable transfectants were selected, and expression of the gene was confirmed by PCR and Western blot analysis. Clones were incubated for 72 hours in serum-free medium (Opti-MEM, Gibco-BRL), the last 4 hours in the presence or absence of 50 μg/mL heparin (bovine lung, Sigma), and processed for secretion studies or purification as described below.

Secretion Studies

Conditioned medium (CM) was collected and concentrated 20 times by using Microset-10 concentrators (Filtron, PolyLabo). Cells were scraped, resuspended in lysis buffer consisting of 10 mmol/L Tris and 5 mmol/L EDTA, and lysed using a syringe fitted with a 25-gauge needle. Large debris, nuclei, and nonlysed cells were removed by centrifugation for 10 minutes at 3000g. The supernatant was centrifuged at 30 minutes at 100 000 g. The pellet was extracted with 1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS in PBS. Cytosolic and membrane/particulate fractions and concentrated CM were analyzed by SDS-PAGE on a 10% gel under reducing conditions and transferred to nitrocellulose membrane (Amersham). The membranes were blocked in 10% nonfat dry milk in Dulbecco-PBS (PBS) plus 0.1% Tween 20 for 1 hour at room temperature and then incubated with anti-myc monoclonal antibody (1:5000) (Invitrogen) for 90 minutes in PBS+5% milk. The membrane was washed 3 times for 10 minutes each in PBS+0.1% Tween and incubated with anti-mouse IgG-peroxidase conjugate for 1 hour at room temperature. After 3 10-minute washes in PBS+0.5% Tween, the membrane was stained with a luminescence detection system (enhanced chemiluminescence, Amersham) according to the manufacturer’s instructions.

Purification

Recombinant FrzA protein was purified using a Ni2+-charged agarose resin (ProBond resin, Invitrogen) under native conditions according to the manufacturer’s protocol. Medium conditioned by heparin-treated cells was collected by using Centriprep-10 concentrators (Amicon) and equilibrated in native binding buffer (20 mmol/L sodium phosphate buffer [pH 7.8] and 150 mmol/L NaCl). The concentrated supernatant was then incubated with resuspended resin at 4°C in a rotating device. After centrifugation at 800g for 2 minutes, the column was washed 3 times with 5 mL of native binding buffer by resuspending the resin, rocking it for 2 minutes, and then separating the resin by centrifugation. The resin was then washed 3 times with 5 mL of phosphate buffer (20 mmol/L sodium phosphate buffer [pH 6] and 500 mmol/L NaCl) with the same procedure. The retained protein was eluted stepwise by application of increasing imidazole concentrations (50, 200, 350, and 500 mmol/L) in elution buffer (20 mmol/L sodium phosphate buffer [pH 6] and 300 mmol/L NaCl). All of the fractions obtained were blotted on a nitrocellulose membrane using a dot blot apparatus. For detection of FrzA, an immunoassay using myc monoclonal antibody (Invitrogen) was conducted as previously described in secretion studies. FrzA protein was detected in the fractions of elution buffer containing 200 mmol/L imidazole. These fractions were pooled and dialyzed overnight against PBS at 4°C, and the protein concentration was calculated with the micro-BCA method (Pierce).

Effect of FrzA on Cell Proliferation

Transient Transfection

BAECs (passage 6) were plated at 2 × 10^4 cells/cm^2 in 6-well plastic dishes (Costar) in serum-containing medium and allowed to attach overnight. The cells were then incubated for 3 hours with 1 μg of plasmid and 3 μL of Superfect reagent (Qiagen) in DMEM/10% CS. After the transfection, the medium was removed and replaced with fresh DMEM/10% CS. Cell counts were determined 3 and 5 days later using a Coulter counter. The culture medium was not replaced during the time course. The transient transfections were conducted either with a control plasmid (pZeoSV coding for the β-galactosidase; Invitrogen) or with the same plasmid containing the FrzA coding sequence in its polylinker region. The efficiency of each transfection was determined by immunostaining the cells for the Sh Ble resistance gene and counting the number of positively stained cells. Briefly, this involved cell fixation in paraformaldehyde, preincubation in blocking serum, and application of the polyclonal antibody against Sh Ble protein (1:1000) (Cayla) for 90 minutes in 5% goat serum, 2% BSA, and 0.2% Tween. A biotinylated anti-rabbit secondary antibody (Amersham) was then applied for 30 minutes at room temperature followed by streptavidin–horseradish-peroxidase complex (Amersham). After 30 minutes at room temperature, sections were rinsed with PBS and visualized by incubation with 0.05% (wt/vol) 3,3′-diaminobenzidine tetrahydrochloride dihydrate. A counterstain of 10% Gill hematoxylin was applied.

Preparation of BAEC CM

Media conditioned for 5 days by cells transiently transfected with the control plasmid (CM-C) or with the plasmid containing the insert (CM-pFrzA) were assayed for their effects on the proliferation of BAECs. BAECs plated at 20 000/cm^2 on 6-well plates (Falcon) were grown in the presence of the CM for 5 days before being harvested and counted. All experiments were repeated at least 3 times.

Effect of Purified FrzA

BAECs were plated at 20 000/cm^2 and BSMCs at 10 000/cm^2 on 12-well plastic dishes (Falcon). After cell attachment, the medium was replaced with 1.2 mL of the mixture of 10% CS plus DMEM with or without purified FrzA. Cells were grown for 2 days in the presence of increasing concentrations of the FrzA-purified fraction before being harvested and counted. All experiments were repeated at least 3 times in duplicate dishes with 3 different preparations of FrzA. Controls consisted of media conditioned by native COS-7 cells concentrated and eluted with the same procedure, as well as the fraction of eluent obtained before and after the peak of FrzA elution. Neither of these control eluents affected cell proliferation.

Results

cDNA Cloning and Sequence Analysis

Differential display was initially used to identify genes differentially expressed between endothelium in vivo and ECs in culture.17 A PCR-amplified cDNA fragment (called Duplaa et al June 25, 1999 1435

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A1) was isolated from endothelium in vivo, cloned, and sequenced. To isolate the full-length cDNA, a cDNA library constructed from RNA isolated from endothelium directly scraped from adult bovine aortas was screened with the A1 cDNA probe. Five independent overlapping cDNA clones were isolated. Together, the clones were found to cover a cDNA sequence of 4.240 bp (Figure 1). The complete cDNA sequence obtained from different overlapping clones contains a single, complete long open reading frame (nucleotides 198 to 1124) and encodes a predicted protein product of 308 amino acids (Figure 1). On the basis of the hydrophobicity profile (data not shown), there is a predicted signal sequence of 29 amino acids at the N terminus with a characteristic composition. Close to the N terminus are 3 polar residues (Gly2–4), and within the leader is a hydrophobic core consisting largely of hydrophobic amino acids (Trp6–Ala22). A polar C-terminal (Gly23–Tyr30) region is also present. Two putative N-glycosylation sites were identified at positions 167 and 257.

While this work was in progress, reports were published defining a novel family of secreted protein related to the frizzled transmembrane protein.15 Bovine FrzA gene was the bovine counterpart of the cloned mouse sFRP1 encoding for proteins presenting 93% of identity. It seemed very unlikely that FrzA is an alternatively spliced gene. A single transcript of ~4.3 kb was detected when a Northern blot of bovine brain and liver total RNA was probed with different cDNA probes that had been used to screen the library. Levels of the transcript were high in the brain and quite low in the liver (Figure 2), observations that were later confirmed by both RNase protection (Figure 4) and in situ hybridization (Figure 5a, 5b, 5k, and 5l). A human orthologue was subsequently cloned presenting 94% identity with the bovine amino acid sequence.16,23,24

Tissue Distribution of FrzA

In an attempt to gain some insight into the potential function of FrzA gene product, we studied the expression pattern of FrzA transcripts in various adult bovine tissues by RNase protection and by in situ hybridization. RNase protection

Figure 2. Northern analysis of bovine FrzA in bovine tissues. Total RNA (30 μg) from the liver and brain tissues was fractionated by gel electrophoresis. RNA sizes were determined by comparison with the ribosomal RNA.
revealed that FrzA mRNA levels were highest in aortic endothelium, heart, spleen, and eye. Lower but significant levels were found in the lung, brain, and kidney. Faint signals were detected in liver, skeletal muscle, and the medial layer of the aorta, and no signal was detected in the aortic adventitia (Figure 3).

To determine which cells were producing FrzA, various adult bovine tissues were examined by in situ hybridization. Our results indicate that FrzA synthesis is highly cell specific. The cortical part of the brain was labeled, with neuronal cells and small vessels strongly positive (Figure 4a and 4b). ECs and SMCs were labeled. FrzA mRNA was expressed in several layers of the retina, with labeling strongest in the inner nuclear layer, where it was localized to astrocytes and ganglion cells (Figure 4c and 4d). In the sclera of the eye, the endothelium and smooth muscle of blood vessels expressed FrzA (Figure 4e and 4f), whereas the connective tissue was negative. Neural cells in the optic disc were not labeled, and FrzA expression was confined to the endothelium of the central artery (data not shown). Expression of FrzA in the heart was restricted to myocytes (Figure 4g and 4h). The skeletal muscle (Figure 4i and 4j) and the liver (Figure 4k and 4l) were both negative for FrzA transcript. In the lung, a high level of labeling was seen in terminal bronchioles, where FrzA transcripts were detected in the epithelium as well as in cells that we suspect are connective tissue macrophages (Figure 4m and 4n). It was notable that not all of the epithelium was labeled; ciliated cells strongly expressed FrzA, whereas Clara cells, which have been described as undifferentiated stem cells, were negative. Interestingly, transcripts were not detected in the large vessels in the lung. In the kidney, the FrzA mRNA was localized in the epithelium of collecting ducts in the medulla (Figure 4o and 4p), whereas the cortical kidney was negative, with no labeling in the glomeruli. The endothelium and the media of the major arteries were mildly positive. In the spleen, FrzA expression was restricted to the red pulp in cells associated with the sinuses (Figure 4q and 4r); these cells could be vascular or lymphatic ECs or macrophages. In the white pulp, the central arteries, capsula rich in smooth muscle cells, and connective tissue were all negative. In general, the labeling pattern observed by in situ hybridization was in agreement with that noted by RNase protection (Figure 3) and indicates a cell-specific pattern of FrzA expression.

**Immunohistochemistry**

Immunohistochemical analysis was used to examine FrzA protein localization in adult bovine tissues. Serially cut, hematoxylin-stained sections of brain, retina, sclera, aorta, lung, and kidney were examined (Figure 5). In general, protein localization was similar to that observed for mRNA by in situ hybridization. In the cortical brain, staining was localized to the neurons and small blood vessels (Figure 5a and 5b). FrzA was localized in the retina to the inner and the outer nuclear layers, with strong labeling of the neuronal cell bodies (Figure 5c and 5d), and displayed a strong immunolabeling in vessels in the choroid and the sclera (data not shown). In the aorta, there was strong immunoreactivity in the endothelium, and a few SMCs in the medial layer were moderately labeled (Figure 5e and 5f). Epithelial cells of the terminal bronchioles in the lung, which showed a strong
Figure 4. In situ hybridization analysis of FrzA transcripts in adult bovine tissues. The first micrograph of each pair (a, c, e, g, i, k, m, o, and q) represents antisense labeling, whereas the second micrograph of each pair (b, d, f, h, j, l, n, p, and r) represents sense labeling. FrzA mRNA is present in the cortical brain (panels a and b), in neurons (n) and blood vessels (bv) and in the retina (panels c and d) in the ganglion cell layer (g), inner plexiform layer (ip), and outer plexiform layer (op), but not in the inner nuclear layer (in), the photoreceptor layer (p), the retinal pigment epithelium (rpe), or the choroid (ch). In blood vessels of the sclera of the eye (panels e and f), the transcripts are in the smooth muscle cells (m) and endothelium (e); l indicates the lumen of the vessel. Cardiac muscle (panels g and h; cm) is highly positive. FrzA is not detected in skeletal muscle (panels i and j; skm), and it is not detected in liver (panels k and l), in either the portal vein (pv) or hepatocytes (h). In the lung (panels m and n), the epithelium of the terminal bronchiole (tbe) is labeled; in kidney medulla (o and p), FrzA is detected in the epithelium of the collecting tubules (cte); in the spleen (q and r), the red pulp (rp) is positive but not the septum (se). Arrowheads indicate FrzA transcript labeling.
signal by in situ hybridization, were also intensely immuno-positive. Interestingly, the blood vessels in the lung were unstained by the FrzA antisera (Figure 5g and 5h). Immunolocalization in the kidney displayed a pattern of FrzA protein expression similar to that seen for mRNA by in situ hybridization, with staining in the epithelium of the collecting ducts (data not shown) and the media and the endothelium of the arteries (Figures 5i and 5j). The 2 antisera yielded similar staining patterns on different bovine tissues; control preimmune serum showed no signal.

Expression of FrzA by ECs In Vitro
Because FrzA was originally identified as differentially expressed between ECs in culture and endothelium in vivo, we examined its expression by ECs in vitro. RNase protection was used to investigate the effect of growth state on endothelial expression of FrzA. ECs, collected by scraping the intimas from freshly obtained bovine aortas, were pooled and plated at low density. FrzA levels were examined after 72 hours, when cells were sparse and proliferating, and after 10 days, when cells were confluent (Figure 6). Sparse BAECs expressed barely detectable levels of FrzA, and expression increased when cells were confluent. There was significant FrzA expression in first-passage ECs, but after 2 additional passages FrzA mRNA levels were undetectable. Culture in serum-free conditions resulted in a strong induction of FrzA in primary BAECs. Serum-free culture conditions, however, did not reinduce FrzA levels in BAECs that had been passaged.

Protein Expression, Secretion, and Purification
To determine whether FrzA was secreted, different protein fractions were examined by immunoblot analysis. FrzA was associated with the plasma membrane but released in the medium by heparin treatment. After 3 days in culture under serum-free conditions, cells were treated for 4 hours with heparin. Then the CM was collected, and the cells were extracted. The majority of the tagged protein was detected in the medium, and the intensity of the band increased after heparin treatment. The protein was not detected in the cytosolic fractions and was barely detectable in the lysates. There was no evidence of partial hydrolysis (Figure 7A). The presence of 6 histidines in the C terminus of FrzA allowed us to purify it from the supernatant of stably transfected COS-7 cells. CM was collected and fractionated on a Ni2+ charged agarose resin column. The major fractions containing FrzA protein were identified by dot blot by probing with myc antibody. FrzA was detected in the 200 mmol/L imidazole fractions (Figure 7B). The purified protein was visualized as a major band by silver-staining SDS-polyacrylamide gel. The identity of the protein was confirmed by immunoblotting with a myc antibody (Figure 7C).

Characterization of FrzA
To investigate its role, studies were conducted to determine whether FrzA would influence EC proliferation in vitro. BAECs were transiently transfected with a plasmid coding either β-galactosidase (control) or FrzA and allowed to grow for 3 or 5 days without a change in media, and cell number was determined. The efficiency of each transfection was determined by immunostaining the cells for the Sh Ble resistance gene and counting the number of positively stained cells. The level of transfection was equivalent between cells transfected with the control plasmid and cells transfected with the plasmid containing the FrzA insert (data not shown), allowing comparison of the effect of FrzA expression on cell proliferation.
Figure 5. Localization of FrzA protein in bovine adult tissues. Sections (5 \( \mu \)m) of paraffin-embedded brain (a and b), retina (c and d), aorta (e and f), lung (g and h), and kidney (i and j) were stained with a rabbit polyclonal antibody directed against a C-terminal peptide of FrzA. The sections were then processed using streptavidin-peroxidase as described in Materials and Methods. Sections in a, c, e, g, and i were incubated with the anti-peptide antisera; those in b, d, f, h, and j were incubated with a nonspecific polyclonal antiserum. There was no staining with the control antiserum in any case (b, d, f, h, and j). Abbreviations are as in Figure 4. Arrowheads indicate FrzA immunostaining. Bar = 200 \( \mu \)m.
Cells transfected with FrzA were growth inhibited when compared with the cells transfected with the control plasmid (Figure 8A). There was a 50% decrease in cell number 5 days after the transfection. Parallel studies revealed a similar decrease in \[^{3}H\]thymidine incorporation (data not shown). Because our studies confirmed that FrzA is a secreted protein, medium conditioned by the FrzA-transfected cells was also assessed for its effect on the proliferation of untransfected BAECs (Figure 8B). Media conditioned for 5 days by cells transfected with the control plasmid (CM-C) or cells transfected with the plasmid containing the insert (CM-FrzA) were added at a final concentration of 25%, 50%, or 75% to BAECs plated at a sparse density. The cells were allowed to grow for 4 days without a medium change, and cell number was determined. CM-FrzA inhibited endothelial growth relative to CM-C. Cell growth was inhibited by 27%, 30%, and 33% at 25%, 50%, and 75% CM, respectively. Parallel studies revealed a similar decrease in \[^{3}H\]thymidine incorporation (data not shown).

Next, we examined the effect of highly enriched fractions of recombinant FrzA on BAEC growth. BAECs were grown for 48 hours in the presence of FrzA, and cell number was determined. FrzA inhibited the proliferation in a dose-dependent fashion. At estimated doses of 1.4 to 14 nmol/L, EC proliferation was inhibited 10% to 60%, respectively (Figure 8C). We also tested the effect of FrzA on BSMCs and showed a significant proliferation inhibition at the same doses (data not shown). As a control, we tested the effect of (1) fractions from CM from heparin-treated native COS-7 cells purified on a Ni\(^{2+}\)-charged agarose resin column and (2) fractions obtained before and after the FrzA peak elution fractions on cell proliferation. These fractions did not affect EC growth (data not shown).

**Discussion**

This work was initially motivated by an interest in identifying genes involved in the induction and maintenance of the quiescent, differentiated phenotype of vascular endothelium. Previous studies have demonstrated phenotypic differences between quiescent endothelium in vivo and their in vitro counterparts. The phenotype of EC in culture is more consistent with cells that have been injured or otherwise activated than cells that are quiescent.\(^{26,27}\) Differential display was used to compare gene expression of quiescent ECs isolated directly from bovine aorta with that of cultured, proliferating ECs. Several cDNA tags expressed predominantly by bovine aortic endothelium in vivo were isolated, but sequence information did not reveal any clue to their possible functions.\(^{17}\)

To isolate the complete cDNA of one of these tags (called A1), we constructed a cDNA library from ECs directly...
scraped from bovine aortas and used the gene tag obtained by differential display to isolate an additional sequence. Sequence homology analyses of this gene revealed that it encodes a predicted protein that contains the characteristic CRD of fz proteins but lacks both the transmembrane and cytoplasmic domains that characterize members of the fz family. Sequence analysis indicated that the protein was secreted. We therefore hypothesized that this protein was a form of the extracellular ligand binding portion of the fz receptor. Western blot analysis of FrzA protein production as a result of transient translation has confirmed that FrzA is secreted.

This gene does not appear to be a splice variant of the longer fz receptor. In the 4 successive library screens, the same sequence was obtained by restriction profile comparison. In a fifth screen, 5 independent clones yielded the identical sequence. Moreover, Northern blot analysis of bovine tissues using probes spanning different regions of the cDNA revealed a single transcript of \( \approx 4.3 \) kb. PCR analysis using different sets of primers spanning the entire cDNA sequence yielded a single band for each primer set (data not shown).

Recently, there have been several reports describing proteins in mouse and human homologous to the Fz CRD called secreted frizzled-related proteins (sFRPs)\(^{15}\) or secreted apoptosis-related proteins (SARP)s.\(^{16}\) SDF5, now referred to as sFRP2 or SARP1, was cloned by signal sequence trapping from a murine bone marrow stromal cell library and is expressed at high levels in lung and kidney.\(^{28}\) Frzb, an orthologue of the mouse sFRP3, was identified in bovine articular chondrocytes.\(^{13}\) Fritz, a CRD-related protein initially cloned from hamster fibroblasts, is highly homologous to Frzb.\(^{29}\) FrzA is the bovine orthologue of the mouse sFRP1 or SARP2. sFRP, the human orthologue, was purified with
hepatocyte growth factor by its heparin-binding capacity from CM by a human embryonic lung fibroblast line.\textsuperscript{23} Interestingly, hSARP2 was isolated as a result of an attempt to clone genes expressed in quiescent but not in proliferating cells. Experiments with SARP2 demonstrated an increased sensitivity of 10T1/2 cells to proapoptotic agent.\textsuperscript{16} Screening for apoptosis-related genes led to the cloning of hsFRP gene, which was suggested to be involved in growth inhibition and in breast tumor development.\textsuperscript{24} The sequence of FrzA is most similar to that of mouse, rat, and Drosophila fz CRD, with \textasciitilde38% identity at the amino acid level and conservation of all 10 cysteine residues.\textsuperscript{1,3,4,13} The CRD motif is also found in a variant of type XVIII collagen.\textsuperscript{30}

To gain some insight into the potential role of FrzA, we studied the expression of FrzA mRNA and protein distribution. FrzA mRNA and protein were expressed widely in adult bovine tissues, yet the cellular localization was quite specific. Immunohistochemical analysis of FrzA protein revealed a pattern of localization that was consistent with the mRNA distribution seen by in situ hybridization. We demonstrated that FrzA is expressed at high levels by epithelium of lung and kidney, by neural elements of the brain and the retina, and by cardiocytes. There was a striking lack of signal in skeletal muscle and liver. In general, a majority of vessels were labeled. In large blood vessels, there was a significant expression in the endothelium, whereas labeling in medial smooth muscle cells was scattered. Expression in microvessel was variable; small vessels in skeletal muscle were not labeled; vessels in all other organs were strongly labeled both in the intima and media. Detection of the protein in the cytoplasm is consistent with its localization in vesicles, as it has been reported for other secreted proteins such as platelet-derived growth factor.\textsuperscript{31} Our description of FrzA transcript distribution at the tissue level is in agreement with that described by RNase assay by Rattner et al\textsuperscript{15} for sFRP1 in mouse adult tissues. Our study, furthermore, has described the expression of FrzA at the cellular level. These data at the level of sequence taken together indicate a high level of conservation and expression of protein, which suggests a functional conservation among the species.

The expression of FrzA was confined to cells that are highly polarized, nonproliferating, and differentiated. A possible role for FrzA in the regulation of growth and differentiation is supported by our in vitro observations. The endothelium of both large and small adult blood vessels does not proliferate and has been documented to have a labeling index of \textasciitilde0.01\%.\textsuperscript{32} BAECs scraped directly from fresh bovine aortas had the highest level of FrzA mRNA. Placement of ECs into culture in the presence of serum, which is known to stimulate proliferation and induce dedifferentiation of EC,\textsuperscript{27} led to reduced FrzA mRNA levels. Repeated passage of the BAECs led to a further reduction in FrzA mRNA until, by passage 5, the transcript levels were nondetectable by RNase protection and could not be reinduced by withdrawal of serum. In addition, BAEC proliferation was reduced by transient expression of FrzA and by culture either in medium conditioned by cells overexpressing FrzA or in the presence of highly enriched FrzA fractions. This effect was not specific to BAECs, because BSMCs were also inhibited by FrzA.

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**Figure 8.** Effect of FrzA on EC proliferation. A, Effect of transient expression. BAECs were plated at 2\times10^4 cells/cm^2 in 6-well plastic dishes. The following day, the cells were transfected overnight in serum-free medium either with the control plasmid (C) or with the plasmid containing the FrzA insert and then replated (day 0) in serum-containing medium. Cell number was determined at 3 and 5 days by counting. B, Effect of medium conditioned by ECs transiently transfected with FrzA on EC proliferation. ECs were plated at 2\times10^4 cells/cm^2. The next day, the cells were rinsed with PBS, and media conditioned for 5 days by cells that had been transfected either with the control plasmid (CM-C) or with the plasmid with insert (CM-FrzA) were added. CM were added at final concentrations of 25\%, 50\%, and 75\%. After 4 days, EC number was determined by counting cells. The experiment was repeated 3 times, with the same results. C, Effect of highly enriched FrzA fractions on EC proliferation. BAECs were plated at 2\times10^4 cells/cm^2 in 12-well plastic dishes. Six hours later, the media were removed and replaced by fresh media containing estimated FrzA concentrations of 0.1, 1.4, 7, 10, and 14 nmol/L. Cell number was determined after 2 days. Data are mean\pmSEM.
Indirect evidence suggests that FrzA binds heparin. Indeed, we demonstrated that heparin releases FrzA in the CM. The highly human homologue of FrzA, hFRP, was purified on a heparin-Sepharose column. As we obtained enriched FrzA fractions from stably transfected COS-7 cells treated with soluble heparin, one could argue that heparin could inhibit the ability of FrzA to suppress BAEC proliferation. Three kinds of experiments were conducted to demonstrate that FrzA influences the growth of vascular cells: transfection experiment, CM assays, and the addition of recombinant purified protein. In the last experiment only, cells were treated with heparin to enhance the free fraction of FrzA protein in the CM, which was purified on a Ni²⁺-charged agarose resin column. Recombinant protein was collected and, during the concentration step, was extensively washed with NaCl before being applied to the column. To remove heparin, different concentrations of NaCl ranging from 0.5 to 1.5 mol/L were used in the washing step in preliminary experiments. Each time, we obtained the same growth-inhibition level (data not shown). In addition, the fact that the transfection experiment (without added heparin in the CM) led to growth inhibition is strong evidence that this effect is due to FrzA. The silver stain of the purified preparation fractions reports the presence of additional bands. Also, we cannot rule out the possibility that the antiproliferative activity present in the FrzA preparation is due to the presence of contaminating cytokine. This is not probable, however, given that fractions from CM from heparin-treated native COS-7 cells and fractions obtained before and after FrzA peak elution fractions did not affect cell growth.

Interestingly, hsFRP, the human orthologue, was found to be regulated during the cell cycle; there was an increase in hsFRP expression in serum-deprived, growth-arrested cells and a decrease in proliferating cells. Moreover, in human breast tumors and in proliferative lesions, hsFRP expression was downregulated. Similarly, although it did not affect cell growth, the overexpression of SARP2 in epithelial tumoral MCF7 cells sensitized the cells to proapoptotic events. Our study reports for the first time that FrzA influences the growth of vascular cells. The mechanism of the EC and SMC growth inhibition is currently under investigation. The expression of Wnt family members in the adult endothelium has not been reported.

The extracellular CRD motif of Fz is thought to serve as a site of ligand binding interaction. The Drosophila fz protein, Dfz2, and other frizzled mammalian members have been shown to bind Wg, a Wnt-1 homologue. The 7 transmembrane domains are theoretically involved in cellular signal transduction. Some insight into the possible role played by FrzA and related proteins is provided by the fact that FrzA, sFRP2, and sFRP3 have been shown to bind and inhibit the effects of members of the Wnt family. Our identification of a secreted truncated form of frizzled receptor protein in the vasculature is intriguing in terms of a potential biological role of this protein in vivo. Other secreted forms of growth factor receptors have been described, including the interleukin 4 receptor, the interleukin 7 receptor, and the fibroblast growth factor receptor. Similarly, secreted forms of adhesion molecule have been described, including the neuronal cell adhesion molecule, the vascular cell adhesion molecule and endothelial leukocyte adhesion molecule. As for these other secreted proteins, FrzA may well act as a competitive inhibitor and therefore a naturally occurring antagonist for the ligand.

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References


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