Primary Structure and Tissue Distribution of FRZB, a Novel Protein Related to Drosophila Frizzled, Suggest a Role in Skeletal Morphogenesis*

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From the ‡Bone Research Branch, National Institute of Dental Research, National Institutes of Health, Bethesda, Maryland 20892, ¶Laboratory of Developmental Biology, Center for Biologics Evaluation and Research, Food and Drug Administration, Bethesda, Maryland 20892, and §Department of Anatomy, Medical School of Zagreb, 41000 Zagreb, Croatia

Articular cartilage extracts were prepared to characterize protein fractions with in vivo chondrogenic activity (Chang, S., Hoang, B., Thomas, J. T., Vukicevic, S., Luyten, F. P., Ryba, N. J. P., Kozak, C. A., Reddi, A. H., and Moos, M. (1994) J. Biol. Chem. 269, 28227–28234). Trypsin digestion of highly purified chondrogenic protein fractions allowed the identification of several unique peptides by amino acid sequencing. We discovered a novel cDNA encoding a deduced 36-kDa protein using degenerate oligonucleotide primers derived from a 30-residue peptide in reverse transcription polymerase chain reactions. Its N-terminal domain showed ~50% amino acid identity to the corresponding region of the Drosophila gene frizzled, which has been implicated in the specification of hair polarity during development. Hydrophathic and structural analyses of the open reading frame revealed the presence of a signal peptide and a hydrophobic domain followed by multiple potential serine/threonine phosphorylation sites and a serine-rich C terminus. Cell fractionation studies of primary bovine articular chondrocytes and transfected COS cells suggested that the protein is membrane-associated. In situ hybridization and immunostaining of human embryonic sections demonstrated predominant expression surrounding the chondrifying bone primordia and subsequently in the chondrocytes of the epiphyses in a graded distribution that decreased toward the primary ossification center. Transcripts were present in the craniofacial structures but not in the vertebral bodies. Because it is expressed primarily in the cartilaginous cores of developing long bones during embryonic and fetal development (6–13 weeks) and is homologous to the polarity-determining gene frizzled, we believe that this gene, which we named frzb, is involved in morphogenesis of the mammalian skeleton.

The discovery and identification of diffusible factors that regulate skeletal morphogenesis have dramatically improved our understanding of the molecular events governing skeletal pattern formation. Genetic studies have confirmed the importance of these differentiation factors in the formation, growth, and maintenance of the skeleton (2). Likewise, nondiffusible molecules, including components of the extracellular matrix and cell surface, are essential to patterning processes. One concept proposed for insect systems postulates that morphogenesis results from the (re)positioning of cells because of inherent characteristics such as differential adhesiveness (3). Whether analogous events occur in mammalian skeletal pattern formation is unknown.

In Drosophila melanogaster, the cuticle contains hairs and bristles arranged in a defined polarity, of which the pattern and orderly alignment reflect the polarity of the wing epidermis (4). Typically, these structures are aligned in parallel and point in the same direction on the body surface. Several genetic loci associated with epidermal cell polarity have been studied. One of the most thoroughly investigated is the frizzled (fz) locus. frizzled encodes an integral membrane protein with seven potential transmembrane domains. This locus is required for cellular response to a tissue polarity signal as well as intercellular transmission of that signal along the proximal-distal wing axis (5, 6). Mutations of the fz locus result in disruption of both cell-autonomous and non-cell-autonomous functions of the fz gene. Strong fz mutations are associated with random orientation of wing hairs. Weaker mutations lead to hair and bristles oriented parallel to neighboring cells, although orientation is random with respect to the body axis (5). In addition, it was recently shown that frizzled regulates mirror-symmetric pattern formation in the Drosophila eye (7).

The homologues frizzled-1 and frizzled-2 (fz-1, fz-2) have been cloned from rat and human, and Northern analysis revealed expression in a wide variety of tissues including kidney, liver, heart, uterus, and ovary (8, 9). Six novel mammalian frizzled homologues have been identified recently (10). Interestingly, each of them seems to be expressed in a distinctive set of tissues during development or postnatally (10). In addition, a Frizzled-like domain has been found in one of the N-terminal noncollagenous domains of α1 (XVIII) collagen (11), although its similarity to other members of the class is comparatively low.

In an effort to identify signaling molecules involved in skeletal patterning and skeletal tissue formation, we used cartilage to isolate highly purified protein fractions with in vivo chondrogenic activity (1). Chondrogenic/osteogenic activity was as-

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† The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) U24163 for human FRZB and U24164 for bovine Frazb.

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1 The abbreviations used are: fz, frizzled; PCR, polymerase chain reaction; RT-PCR, reverse transcription-PCR; kb, kilobase pair(s); PBS, phosphate-buffered saline; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid.
sawed in vivo by subcutaneous implantation of ethanol-precipitated protein fractions in rats (12); implants were recovered after 10 days and analyzed histologically for alkaline phosphatase activity. Trypsin digestion of the bioactive purified fractions and isolation of the resulting peptides allowed identification of several unique peptides by amino acid sequencing. Starting from a 30-amino acid peptide, we identified a cDNA construction of several unique peptides by amino acid sequencing. The resulting peptides allowed identification of several regions that were separated by reverse phase (17), and the sequence of the purified protein fractions in rats (12); implants were recovered after 24 h at 42°C in 6 M urea, 1 M thiourea, and 3% CHAPS (U.S. Biochemical Corp.). All sequencing of both strands was done using the dideoxy chain termination method (21) and Sequenase version 2.0 DNA polymerase according to the manufacturer's instructions (U.S. Biochemical Corp.). The sequencing data were obtained by primer walking and from subclones of restriction fragments into pBluescript SKII (Stratagene). Once the bovine cDNA was isolated, PCR was used to generate a 1-kb fragment containing XhoI sites of pET-28a (+) XhoI site of pET-28a (+) XhoI site of pET-28a (+) XhoI site of pET-28a (+) XhoI site of pET-28a (+). The bovine open reading frame fragment was subcloned in the proper orientation into the appropriate plasmid vector (pET-28a (+)). Plasmids harboring hybridizing to both oligonucleotides were further purified using standard affinity chromatography (QIAGEN, Inc.) was performed using the cobalt-nickel-nitrilotriacetic acid (Ni-NTA) chromatography (QIAGEN, Inc.) was performed using the cobalt-nickel-nitrilotriacetic acid (Ni-NTA) chromatography (QIAGEN, Inc.) was performed using the cobalt-nickel-nitrilotriacetic acid (Ni-NTA) chromatography (QIAGEN, Inc.) was performed using the cobalt-nickel-nitrilotriacetic acid (Ni-NTA) chromatography (QIAGEN, Inc.).
Frizzled-related Protein in Skeletal Development

FIG. 2. A, deduced amino acid sequences of a cDNA clone of bovine and human frzb. The predicted 21-amino acid signal peptide is boxed. *, a potential N-linked glycosylation site. The putative transmembrane domain is delineated by a bold underline. B, hydropathy analysis of human FRZB from deduced amino acid sequence. The plot was generated by the GeneWorks™ program using the paradigm of Kyte and Doolittle. Hydrophobic residues are in the upper part of the graph. The arrowhead at the N-terminal depicts the potential signal peptide. The putative transmembrane domain is indicated by a downward arrow. N, C, and P are N-glycosylation, CK2 phosphorylation, and protein kinase C phosphorylation sites, respectively. The stippled bar underneath the plot represents the Frizzled-like domain.

1) coupled to keyhole limpet hemocyanin through a C-terminal cysteine. The resulting antisera were screened and titered in immunoblots using the Western-Light Plus Kit® (Tropix, Inc.) according to the manufacturer’s protocol. Briefly, the membranes were blocked overnight in blocking buffer (BF) consisting of 0.6% 1-BLOCK™ (Tropix, Inc.) in PBS and 0.1% Tween 20. The antisera was diluted from 1:250 to 1:10,000 in BF. The membranes were washed three times for 5 min in BF after each incubation step. The membranes were then incubated with the secondary antibody at a dilution of 1:20,000 for 30 min, followed by AVIDIX™ (enzyme conjugate) incubation for 20 min. Blots were developed using the CSPD™ chemiluminescent substrate (Tropix, Inc.) and exposed to Eastman Kodak Co. XAR-5 films for 1–10 min.

RESULTS

frzb Encodes a Protein with Regional Homology to Frizzled—To clone cDNAs corresponding to tryptic peptide 323, RT-PCR was performed using bovine articular cartilage poly(A) RNA as a template. This yielded a 90-base pair DNA fragment encoding the proper peptide sequence (Fig. 1, dashed underline). Screening of a bovine articular cartilage cDNA library using two different 30-mer oligonucleotides designed from the 90-base pair fragment identified a 2.4-kb clone that hybridized to both oligonucleotides. This clone contained a...
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Fig. 3. Amino acid sequence comparison of the N-terminal domain of bovine and human FRZB: homology with rat Fz-1 (8) and Drosophila Frizzled (6). Identical residues are denoted by shaded boxes. Gaps (−) are introduced in the sequence to optimize alignment, and asterisks (*) denote sites of conserved cysteine residues. The numbers on the right indicate amino acid residues for each protein.

Fig. 4. Protein expression, antibody characterization, and compartmentalization studies. A, Frzb fusion protein was purified from bacterial inclusion bodies using a Ni-NTA column. Aliquots of protein fractions were run on an SDS-polyacrylamide gel according to Laemmli (29) and stained with Coomassie Blue (lane 1, eluate). Left, molecular weight standards in kDa (lane MW). Membrane fractions from cell lysates were prepared as described under “Materials and Methods.” The immunoreactivity of this protein product was tested by immunoblotting using antiserum N374-PEP (lane 2). B, Nontransfected and frzb-transfected COS1 cells were lysed and processed as described under “Materials and Methods.” The various fractions were separated on SDS-polyacrylamide gel electrophoresis gels under reduced conditions and analyzed in immunoblots. Lane 1, conditioned medium; lane 2, cytosol; lane 3, 1% Triton X-100; lane 4, Triton X-100/urea/SDS extract. C, Primary bovine articular chondrocytes were grown to confluence. The cultures were subsequently maintained for 48 h in serum-free conditions in the presence or absence of dextran sulfate (250 μg/ml); supernatants were collected, and cells were lysed, processed, and analyzed in immunoblots as above. Lane 1, membrane fraction from cell lysates with dextran sulfate; lane 2, without dextran sulfate; lanes 3 and 4, supernatants of samples shown in lanes 1 and 2, respectively.

single open reading frame with two separate consensus polyadenylation sites and a poly(A) tail (Fig. 1). A 1.3-kb clone contained a single polyadenylation signal, a short poly(A) tail, and a short 5′-noncoding region. Three other clones lacked the poly(A) tail but contained longer 5′-ends. Because Northern analysis using a bovine cDNA probe revealed mRNA expression in placenta (data not shown), we screened a human placenta cDNA library to isolate the human orthologue. Four clones ranging from 1.3–1.6 kb were analyzed, and all contained the same open reading frame. All clones contained a consensus translation initiation site (24) and in-frame termination codon situated 144 base pairs upstream of the start methionine (Fig. 1). The difference in size between the bovine and human cDNA inserts (2.4 kb versus 1.3 kb) is due to a longer 3′-untranslated sequence in the bovine clone (Fig. 1). Based on sequences from these overlapping cDNA clones, the predicted sizes of both the human and bovine protein is 325 amino acids (Fig. 2A).

The bovine and human amino acid sequences are 94% identical. The deduced protein sequence of both the human and bovine cDNA revealed at least four structural domains (Fig. 1; Fig. 2, A and B). An N-terminal hydrophobic stretch of 25 amino acids immediately downstream of the initiation methionine likely represents a signal peptide (25). A second hydrophobic stretch of 24 amino acids (residues 75–98), which could represent a transmembrane domain, is followed by a region with several potential serine/threonine phosphorylation sites and a serine-rich C-terminal domain (residues 301–325). Both homologues contain an N-linked glycosylation site at Asn-49, which is N-terminal of the putative transmembrane domain. A potential C-terminal glycosylation site in the bovine protein was not present in the human homologue.

A search of the GenBank® data base using the basic local alignment search tool network service at the National Center for Biotechnology Information (26) indicated that Frzb has significant identity (~50%) in the N-terminal region (from amino acid 35–147) to Drosophila Frizzled and rat Fz proteins (Fig. 3). The homologous region begins shortly after the cleavage site of the predicted signal sequence. The 10 cysteines in this region are conserved.

Frzb Is a Membrane-associated Protein—Frzb fusion protein containing six N-terminal histidines was purified from bacterial inclusion bodies using Ni-NTA affinity chromatography. The affinity-purified protein was visualized as a major band following Coomassie Blue staining after SDS-polyacrylamide gel electrophoresis (Fig. 4A, lane 1). The identity of the fusion product was verified by immunoblotting using the T7 monoclonal antibody (data not shown). Antiserum N374-PEP (see “Materials and Methods”) detected a band migrating at the same apparent molecular weight on a Western blot (Fig. 4A, lane 2).

Immunoblots of protein fractions obtained from cultured cells were performed to investigate the distribution of Frzb. Experiments were done in COS1 cells transfected with pfrzb. As shown in Fig. 4B (lane 4, transfected), the urea/SDS/Triton X-100 extract of the membrane pellet contained most of the frzb protein. No protein was detected in the supernatants of the transfected cells (Fig. 4B, transfected, lane 1). No protein was detected in untransfected cells (Fig. 4B, control).

Because the protein sequencing data were obtained from partially purified protein preparations of bovine articular cartilage extracts, immunoblots were also performed on supernatants and cell extracts of primary bovine articular chondrocyte cultures. Again, most of the protein was detected in the membrane-associated fractions (Fig. 4C, lane 1). The addition of dextran sulfate (250 μg/ml) did not change this distribution (Fig. 4C, lanes 2 and 4).

Frzb Displays a Dynamic Expression Pattern in Developing Limbs—Serial sections of human embryos representing various stages of development were used for in situ hybridization to
explore the pattern of frzb expression during embryonic development. Between 6 and 13 weeks, no hybridization was detected in most organs, including kidney, heart, muscle, intestine, liver, brain, and lung (data not shown). In contrast, strong hybridization was seen in the developing appendicular skeleton. At 6 weeks, frzb transcripts were clearly visible surrounding the early cartilaginous rudiments of the developing limbs, as shown in the distal parts of the upper limb (Fig. 5). Hybridization is apparent between neighboring areas of cartilaginous condensation in developing long bones. Subsequently, expression appears within the cartilaginous cores of developing long bones. This is apparent in the proximal parts of the upper limb, which are more advanced in developmental stage than the distal parts (Fig. 5). In addition, frzb was detected in the cartilage anlagen of several craniofacial bones (data not shown) and the epiphysial ends of the rib cage (Fig. 5), whereas no signal was detected in the vertebral bodies at 6 weeks. At 13 weeks, frzb transcripts were present in early chondroblasts of the tarsal bones of the foot, the carpal bones of the hand, and the epiphysis of long bones (Fig. 6, I and II). A striking feature of the expression pattern at this developmental stage was the presence of a graded distribution, most prominent in the phalanges, was paralleled by the protein distribution (Fig. 7).

DISCUSSION

To characterize factors responsible for cartilage and bone inductive activity in articular cartilage (1), we isolated a protein fraction containing potent bone and cartilage inductive activity. Edman analysis of peptides obtained by tryptic digestion of the bioactive fraction allowed us to identify novel genes by means of RT-PCR. One of the cDNAs obtained using a PCR product encoding a 30-amino acid peptide as a hybridization probe contained an open reading frame encoding a protein of Mr ≈ 36,200, which agrees well with the molecular weight of the highly purified gel-eluted protein preparations described previously (1). mRNA blots reveal a major signal at 2.4 kb, which is consistent with the size of the bovine cDNA. This clone contained an additional polyadenylation signal that could give rise to the 1.7-kb message on Northern analysis. Edman analyses of four additional peptides in the bioactive protein preparation were consistent with the sequence deduced from the cDNA. Finally, this cDNA was found by independent screening of two different cDNA libraries. These data, together with the hybridization signals obtained in genomic Southern blots (data not shown), provide strong evidence for the existence of frzb in diverse species. The relationship of Frzb protein to chondrogenic activity in the purified fractions of cartilage extracts is unclear. The presence of the protein in these fractions may be coincidental and thus a serendipitous finding.

The presence of a putative signal peptide suggests that Frzb enters the secretory pathway. A hydrophobic 24-amino acid stretch in the N-terminal region suggests the possibility of a transmembrane domain. The C-terminal region of the protein contains several potential serine/threonine phosphorylation sites (7 in bovine and 9 in human) and a region of high serine and threonine content (10 of the last 26 residues). Consequently, this molecule is a potential target for protein phosphorylation via serine/threonine kinases. In addition, subcellular
Fractionation experiments further suggest that Frzb is membrane-associated. The cysteines in the molecule do not seem to form intermolecular disulfide bridges, because the size of Frzb protein in highly purified cartilage preparations did not change in Western blots of SDS-polyacrylamide gel electrophoresis separations performed under nonreducing conditions after reduction and alkylation (data not shown).

A GenBank database search revealed striking sequence similarity of the N-terminal domain of frzb to the Drosophila polarity gene frizzled (6) and its mammalian homologue, the rat fz-1 gene (8). The homologous domain of about 110 amino acids corresponds to the suggested extracellular domain of the frizzled gene products. Unlike the frizzled and fz gene products, Frzb lacks the seven transmembrane domains found in a large family of G-protein-coupled membrane receptors (10). Molecular structural analysis, together with our cell fractionation data, suggests that a 24-amino acid hydrophobic stretch within this domain might provide a membrane-anchoring function. Genomic sequence analysis revealed that in both Drosophila frizzled and frzb, the homologous domain is encoded by the first exon. This suggests a common ancestry and function. Recently, a similar (though less highly conserved) cysteine-rich Frizzled-like domain has been found in one of the N-terminal noncollagenous domains of α1 (XVIII) collagen (11), supporting the notion that the frizzled-like sequence is a conserved motif.

The basic form and pattern of the skeleton derived from lateral plate mesoderm are first recognizable when mesenchymal cells aggregate into regions of high cell density called condensations. They subsequently differentiate into cartilage and bone and continue to grow by cell proliferation, cell enlargement, and matrix deposition. Genetic studies have demonstrated that disruption of these condensations results in disturbed skeletal phenotypes (for review see Ref. 2). In humans, limb development takes place over a 4-week period from the 5th to 8th week (27). The upper limbs develop slightly in advance of the lower limbs, although by the end of the period of limb development, the two limbs are nearly synchronized. The most proximal parts of the limbs develop somewhat in advance of the more distal parts. In the developing human limb bud, frzb appears between and around the early cartilaginous condensations of the limb. Subsequently, transcripts are detected within the cartilaginous cores of the developing skeleton. The expression pattern in the developing long bones is graded; the highest levels appear in chondroblasts of the epiphysis and decrease toward the primary ossification center. Surprisingly, at 13 weeks of embryonic development, the expression of this gene was not observed in several layers of chondrocytes lining the joint surface of the phalanges (Fig. 6I), with a sharp delineation of the expression of frzb at the junction between the superficial (closer to the joint surface) and deeper layers of chondrocytes of the developing long bones. Immunostaining reflected this expression pattern at the protein level (Fig. 7).

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3 J. T. Thomas and F. P. Luyten, unpublished observations.
These findings provide evidence that in early stages of embryonic development, there might already be distinct differentiation pathways for articular and epiphysial chondrocytes. No frzb transcripts were detected in vertebral bodies at any stage of skeletal development up to 13 weeks. These observations suggest distinct molecular pathways for specification of the axial (derived from paraxial mesoderm) as opposed to the appendicular (derived from lateral plate mesoderm) skeleton, as has been suggested previously (1, 28).

Polarity determination is thought to be a crucial step in morphogenesis and pattern formation. The homology of Frzb to Frizzled, together with a unique spatial and temporal expression pattern in developing skeletal tissues, suggests that this newly discovered protein may play a role in skeletal morphogenesis.

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FIG. 7. FRZB immunostaining in the developing human phalanges at 13 weeks. A and C, preimmune antiserum staining; B and D, N374-PEP antiserum staining. Positive staining (brown-black) is detected in the epiphysial chondrocytes of the phalanx. No staining is seen in several layers close to the joint surface or in the chondrocytes close to the primary ossification center. Magnification: A and B, ×100; C and D, ×400.
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