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Fdp, a New Fibrocyte-derived Protein Related to MIA/CD-RAP, Has an *in Vitro* Effect on the Early Differentiation of the Inner Ear Mesenchyme*

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During the course of a study aimed at isolating transcripts specifically or preferentially expressed in the inner ear, we identified a novel gene, encoding a fibrocyte-derived protein, that we named *Fdp*. *Fdp* is predicted to be a secreted 128-amino acid protein, which is highly homologous to the melanoma-inhibiting activity/cartilage-derived retinoic acid-sensitive protein (MIA/CD-RAP), a cartilage-specific protein also expressed in several tumors. *Fdp* and MIA/CD-RAP thus define a new family of proteins. *Fdp* is expressed from embryonic day 10.5 in the mesenchyme surrounding the otic epithelium. During development, these cells progressively aggregate, condense, and differentiate into cartilaginous cells forming the otic capsule, which no longer expresses *Fdp*, and into fibrocytes surrounding the epithelia, which strongly express *Fdp*. In order to address the function of *Fdp*, we developed an *in vitro* antisense oligonucleotide approach using microdissected periotic mesenchyme micromass cultures, and showed that *Fdp* antisense oligonucleotide treatment results in a significant reduction in chondrogenesis. Our results demonstrate that *Fdp* plays a role in the initiation of periotic mesenchyme chondrogenesis. Accordingly, *Fdp* and its human ortholog *FDP*, which map to chromosome 2 and band 20p11, respectively, could be candidate genes for forms of deafness associated with malformations of the otic capsule.

Identifying genes specifically or preferentially expressed in the inner ear is a powerful approach for deciphering the molecular basis of the development and function of this sensory organ. It is also a fruitful approach for isolating the genes involved in hereditary deafness, which affects about 1 individual in 1000 at birth (1). In mammals thus far, this strategy has resulted in the identification of six genes. Initially, the genes encoding α - and β -tectorin were isolated upon the purification of their corresponding proteins (2); mutations in *TECTA*, en-

coding α -tectorin in humans, are responsible for a form of nonsyndromic deafness (isolated deafness) (3–5). Recently, using another strategy based on cDNA subtractive methods, four other genes were isolated, which encode cochlin, otogelin, otoconin-95, and otoferlin (6–9). With the exception of the gene encoding otoconin-95, the major component of the biominerals of the vestibular apparatus (8), mutations in these three other genes have been shown to be responsible for some forms of deafness (9–11). These genes are expressed in a variety of inner ear cell types. Cochlin is expressed by cells surrounding the auditory and vestibular nerve fibers (10); α - and β -tectorins and otogelin are non-collagenous components specific to the acellular membranes of the inner ear, which cover the neuroepithelia, and are expressed by the non-sensory neuroepithelial cells (2, 7); and otoferlin is preferentially expressed by the sensory hair cells (9).

Using a subtractive cDNA approach on mouse cochlea, we have isolated and characterized a new gene preferentially expressed in the inner ear, which we named *Fdp* for fibrocyte-derived protein. The expression pattern of *Fdp* during inner ear development, and its inhibition *in vitro* using an antisense oligonucleotide, suggest that *Fdp* could play a role in the early chondrogenesis of the periotic mesenchyme.

EXPERIMENTAL PROCEDURES

Cloning of the Full-length *Fdp* cDNA—Primers (see below) for rapid amplification of cDNA ends (RACE)¹ using 2-day-old mouse oligo(dT)-primed cochlear cDNA, were designed from the 160-base pair (bp) cDNA subtracted clone (cDNA position 204–362), according to the Marathon cDNA protocol (CLONTECH). Amplifications were carried out using the Expand long template PCR *Taq* polymerase mix (Roche Molecular Biochemicals). 3' extension using the primer L136 (5'-GATC-TATGTTTACTCCAAGCTGGT-3') (cDNA position 204), and 5' extension using the primer U1 (5'-GATCTCCTTGGTGGCCTCCTGGTA-3') (cDNA position 362) exclusively resulted in PCR products of 750 and 361 bp, respectively, that were individually cloned and sequenced.

DNA Sequencing and Analysis—DNA sequencing was carried out using the PRISM[®] Ready Reaction DyeDeoxy[®] terminator cycle sequencing kit (PerkinElmer Life Sciences) and an ABI model 377 DNA Sequencer (PerkinElmer APB Division).

RT-PCR Analysis—Total RNA from 2-day-old or adult mouse tissues, total E9.5, E10.5, and E11.5 embryos, and dissected E10.5 periotic mesenchymal cells (about 250,000 cells) was prepared by the guanidium isothiocyanate procedure. Gestational age was estimated by the

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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) AF243504 (*Fdp*) and AF243505 (*FDP*).

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¹ The abbreviations used are: RACE, rapid amplification of cDNA ends; PCR, polymerase chain reaction; RT-PCR, reverse transcription polymerase chain reaction; bp, base pair(s); kb, kilobase(s); E, embryonic day; P, postnatal day; MIA, melanoma inhibitory activity; CD-RAP, cartilage-derived retinoic acid-sensitive protein; BAC, bacterial artificial chromosome; FGF, fibroblast growth factor; S-GAG, sulfated glycosaminoglycan.

vaginal plug method, with the day of plug occurrence designated as day 1 (E1). Reverse transcription was performed using 250 ng of total RNA (except for the E10.5 periotic mesenchyme, where all of the prepared RNA extracted from about 250,000 cells was used), a random hexamer mix as the primer, and the SuperScript[™] II RNase H⁻ reverse transcriptase (Life Technologies, Inc.). One tenth of the reaction product was PCR-amplified, in a total volume of 50 μ l using the primers L136 (see above) and L511 (5'-GGAGTCAGAGCTGGGGAGAATGA-3') (cDNA position 736). The primers derived from the ubiquitously expressed *Hprt* gene (GenBank[™] accession no. J00423), *Hprt.F* (5'-GCTGGTGAAAAGGACCTCT-3') (cDNA position 576) and *Hprt.R* (5'-CACAGGACTAGAACACCTGC3') (cDNA position 813), were used in the control reaction. PCR amplification was carried out for 35 cycles under standard conditions using the Qiagen *Taq* polymerase. For *Fdp*, amplification specificity was verified by Southern transfer and hybridization of the amplified products with the internal oligonucleotide U1 (see above).

In Situ Hybridization—Whole Swiss E17.5 mouse embryos or 4-day-old mouse heads were fixed for 4 h in 4% paraformaldehyde at 4 °C, embedded in sucrose 20% overnight at 4 °C, and frozen in Tissue-Tek (OCT) using liquid nitrogen. Sagittal and parasagittal cryosections (10 μ m) were collected and treated for hybridization as described by Schaeren-Wiemers and Gerfin-Moser (12). The 160-bp subtracted cDNA fragment was cloned into the pGEM-4Z vector (Promega). Sense and antisense RNA probes were labeled with digoxigenin-11-UTP, and the hybridization signal was detected by sheep anti-digoxigenin antibodies coupled to alkaline phosphatase. The sense riboprobe was used in parallel experiments as a negative control.

Fdp Antisense Oligonucleotide Assays on High Density Culture of E10.5 Periotic Mesenchyme + Otic Epithelium—Periotic mesenchyme + otic epithelium were isolated from E10.5 C57BL/6 mouse embryos as described by Frenz and van De Water (13), and cultured in Ham's F-12 medium in the presence at a concentration of 60 ng/ml or absence of oligonucleotides. The culture medium with the added oligonucleotides was changed daily. After a culture period of 3 days, sites of mesenchymal condensation, which precede cartilage development, were counted and used as an early indicator of chondrogenesis (13). After a culture period of 7 days, the cells were fixed, then stained with Alcian Blue 8GX, pH 1.0. Binding of matrix-bound Alcian Blue stain was measured spectrophotometrically and also used as an indicator of chondrogenesis (13). The following oligonucleotides were used: *Fdp* antisense (5'-CAATATCCTTGCCAT-3') (cDNA position 23); *Fdp* sense (5'-ATG-GCAAGGATATTG-3') (cDNA position 23); β -globin (5'-AGTCCCGT-TACCAT-3'); and random (5'-TACAGCTACTTACCT-3'). The specificity of the selected oligonucleotides was confirmed by sequence comparisons with the NCBI GenBank[™] data base using the BLAST algorithm.

Each experiment performed included one untreated culture, one or two antisense oligonucleotide treated-culture(s), one or two sense oligonucleotide treated-culture(s), and one or two random or β -globin oligonucleotide-treated culture(s). A total of four independent experiments were performed, which included five antisense oligonucleotide treated-cultures, six sense oligonucleotide treated-cultures, three random oligonucleotide treated-cultures, and five β -globin oligonucleotide-treated cultures.

Cloning of the Fdp and FDP Genes—To determine the genome structure of *Fdp*, a 129sv mouse genomic bacterial artificial chromosome (BAC) library (Genome System) was screened by PCR using the primers U1 and L136 (see above). 1 μ g of DNA from the positive BAC clone 96J02 was fully digested by *Sau*3A and cloned into a *Bam*HI-linearized puc18 vector. Three contiguous subclones were found to contain the first three *Fdp* exons by hybridization with the *Fdp* cDNA as a probe. Their sequences were compared with that of the *Fdp* cDNA to determine the limits of the first two exons as well as the 5' limit of the third exon. The 3' limit of the third exon and the 5' limit of the fourth exon containing the entire 3'-untranslated region, were determined by direct sequencing of the BAC DNA.

To determine the genome structure of *FDP*, a human BAC genomic DNA library (Genome System) was screened by PCR using primers derived from the sequence-tagged site WI-16380, presenting with 67% homology to the *Fdp* 3'-untranslated region. 4 μ g of DNA from the positive BAC clone 95H02 were sonicated to generate random fragments between 200 and 1000 bp in length. These fragments were end-filled using Klenow DNA polymerase and subsequently cloned into a *Sma*I-linearized and dephosphorylated M13mp18 vector. *FDP*-containing subclones were isolated by hybridization screening, using a probe containing the *Fdp* cDNA, and sequenced.

Mouse Chromosomal Localization of Fdp—A *Msp*I restriction fragment length polymorphism was detected between the C57BL/6 and SEG mouse strains in the 3'-untranslated region of *Fdp* by the hybridization of a *Fdp* cDNA probe to a Southern blot containing genomic

DNA of both strains. Bands of 3 and 5.5 kb were detected in C57BL/6 and SEG genomic DNA, respectively. The polymorphic *Msp*I site (cDNA position 569) was identified by PCR amplification of mouse genomic DNA from both strains using the primers U245 (5'-AAGAAGTG-GAAATAACTG-3') (cDNA position 446) and L892 (5'-TACCTAACAT-TCTTCT-3') (cDNA position 892), and *Msp*I digestion of the amplified products. Using this polymorphism, the segregation of *Fdp* was compared with that of marker loci from chromosome 2, the region homologous to chromosome band 20p11 containing the human ortholog, *FDP*. These marker loci were previously typed in backcross progeny of the C57BL/6 and SEG strains of the European Collaborative Interspecific Backcross panel (14).

RESULTS

Isolation and Analysis of the Fdp cDNA—Using a PCR amplification-based approach, we recently generated a subtracted mouse cochlear cDNA library (8). From this library, we identified a 160-bp clone preferentially expressed in the inner ear (see below). 3' and 5' RACE-PCR experiments were performed to reconstitute a full-length cDNA of 918 bp (see "Experimental Procedures") (Fig. 1A). A transcript of a similar size was detected on Northern blots using the full-length cDNA as a probe (data not shown). In reference to its site of expression, we named the corresponding gene *Fdp*, for fibrocyte-derived protein. The translation initiation site was identified by the presence of an adequate Kozak consensus sequence GGAAGATGG at position 9 (15). The initiation codon was preceded by a 8-bp 5'-untranslated region, and followed by a 384-bp open reading frame and a 518-bp 3'-untranslated region. The predicted *Fdp* protein sequence starts with a 18-amino acid sequence rich in uncharged and hydrophobic residues, characteristic of a signal peptide (Fig. 1A) (16). Its cleavage would leave His-19 as the N-terminal amino acid of a putative 110-residue mature protein, with a predicted molecular mass of 11 kDa. No evidence of either a transmembrane domain or a glycosylphosphatidylinositol anchorage sequence (17) was found in the deduced amino acid sequence, indicating that this protein is likely to be secreted. It did not contain a glycosylation consensus site, or any previously identified protein domains.

GenBank[™] sequence comparison studies of the *Fdp* cDNA showed that it was approximately 65% homologous to the human, mouse, rat, and bovine *MIA/CD-RAP* cDNA (18–20), suggesting that *Fdp* and *MIA/CD-RAP* may form a new gene family. The *Fdp* protein sequence showed 44% identity and approximately 80% similarity with *MIA/CD-RAP* (Fig. 1B). The four cysteine residues present in all *MIA/CD-RAP* orthologous proteins, which have been suggested to be involved in the formation of two intramolecular disulfide bonds (18), were also conserved in *Fdp* (Fig. 1B). No noticeable amino acid conservation between *Fdp* and *MIA/CD-RAP*, which could be indicative of a protein domain, was found.

Expression Analysis—The expression pattern of *Fdp* was studied by RT-PCR and *in situ* hybridization at different stages of development. After birth (Fig. 2A), RT-PCR experiments showed a strong *Fdp* expression in the cochlea, and a weak expression in the eyes and in cartilage from the clavicle, femur, and cranium. A very faint signal was also detected in the brain. No expression was detected in the liver, lung, skeletal muscle, heart, intestine, or testis. RT-PCR analysis of total embryos throughout development demonstrated that *Fdp* is expressed from E10.5 onward (Fig. 2B). Finally, the embryonic inner ear expression of *Fdp* was analyzed in microdissected periotic mesenchyme. *Fdp* was amplified by RT-PCR from this tissue at E10.5 (Fig. 2A).

Localization of the *Fdp* transcript in the inner ear was then studied by *in situ* hybridization at different stages of development (see "Experimental Procedures"). From E11.5 to E16.5, no signal could be detected (data not shown), even in the periotic mesenchyme where *Fdp* was detectable by RT-PCR (see above),

A

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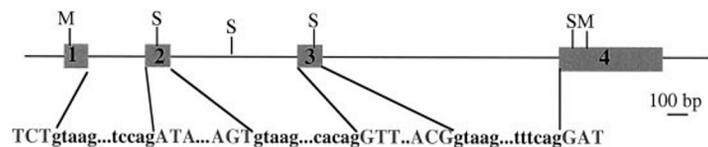
gaaggaagATGGCAAGGATATTGATCTTTTGGCTTGGGGCCCTTGTGGTTCTATGTGCCG 60
      M A R I L I L L L G G L V V L C A G 18
GGCATGGTGTATTTATGGATAAACTTCTTCTAAGAAGTTGTGTGCGGATGAGGAGTGTG 120
      H G V F M D K L S S K K L C A D E E C V 38
TCT↓ATACTATTCTCTGGCAAGAGCACAGGAAGATTACAATGCCCCAGACTGTAGGTTCA 180
      Y T I S L A R A Q E D Y N A P D C R F I 58
TCGATGTCAAGAAAGGCAGCAGATCTATGTTTACTCCAAGCTGGTAACAGAAAACGGAG 240
      D V K K G Q Q I Y V Y S K L V T E N G A 78
CTGGAGAGTTTTGGGCTGGCAGT↓GTTTATGGTGACCACCAGGATGAGATGGGAATTGTAG 300
      G E F W A G S V Y G D H Q D E M G I V G 98
GTTATTTCCCCAGCAACTTGGTGAAGGAGCAGCGTGTATACCAGGAGGCCACCAAGGAGA 360
      Y F P S N L V K E Q R V Y Q E A T K E I 118
TCCAACCACG↓GATATTGACTTCTTCTGTGAATAAgaattaataaaacagcagataaa 420
      P T T D I D F F C E 128
acagaaacaccagtgatgaagaagagaagaagtggaaataactgaacctgtgtatctgtga 480
      ccttctctggcttatttggctggcaggaggttgatcttgaaggtgctaagatatggaaat 540
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      gttttgtactcataaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaa 916
    
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FIG. 1. Structure and sequence of *Fdp*. *A*, *Fdp* cDNA and deduced amino acid sequence. The arrows indicate exon-intron boundaries. The signal peptide is underlined. *B*, multiple alignment of the orthologous *Fdp*, *FDP*, and *MIA/CD-RAP* deduced protein sequences using the pileup program (GCG package). The identical residues are boxed in gray when present in at least three out of six sequences. The four conserved cysteine residues between the *Fdp*, *FDP*, and *MIA/CD-RAP* sequences are indicated by an asterisk. *C*, structure of *Fdp*. The four boxes represent the *Fdp* exons. Intronic sequences are in lowercase, exonic sequences in uppercase. The intron-exon boundaries are in accordance with the splice site consensus sequences (49). *M*, *MspI* restriction site. *S*, *Sau3A* restriction site.

B

human FDP	1	MAR	---	I	L	L	F	L	P	G	L	V	A	V	C	A	V	H	G	I	F	M	D	R	L	A	S	K	K	L	C	A	D	E	36								
mouse Fdp	1	MAR	---	I	L	L	L	L	G	L	V	V	L	C	A	G	H	G	V	F	M	D	K	L	S	S	K	K	L	C	A	D	E	36									
bovin MIA	1	M	A	M	S	L	V	F	L	G	V	-	V	L	L	S	A	F	E	G	P	S	A	G	E	R	P	M	P	K	L	A	D	R	K	M	C	A	D	E	39		
human MIA	1	M	A	R	S	L	V	C	L	G	V	-	I	L	L	S	A	F	E	S	G	P	V	R	E	G	P	M	P	K	L	A	D	R	K	L	C	A	D	E	40		
rat MIA	1	M	V	C	S	P	V	L	L	G	I	-	V	I	L	S	V	F	E	S	G	L	S	R	A	D	R	A	M	P	K	L	A	D	R	K	L	C	A	D	E	39	
mouse MIA	1	M	V	W	S	P	V	L	L	G	I	-	V	V	L	S	V	F	E	S	G	P	S	R	A	D	R	A	M	P	K	L	A	D	R	K	L	C	A	D	E	39	
human FDP	37	C	V	Y	T	I	S	L	A	S	A	Q	E	D	Y	N	A	P	D	C	R	F	I	N	V	K	K	G	Q	I	V	V	Y	S	K	L	V	K	E	N	76		
mouse Fdp	37	C	V	Y	T	I	S	L	A	R	A	Q	E	D	Y	N	A	P	D	C	R	F	I	N	V	K	K	G	Q	I	V	V	Y	S	K	L	V	T	E	N	76		
bovin MIA	40	C	S	H	P	I	S	M	A	V	A	L	O	D	Y	V	A	P	D	C	R	F	L	T	I	H	G	O	V	V	Y	F	S	K	L	-	-	K	G	77			
human MIA	41	C	S	H	P	I	S	M	A	V	A	L	O	D	Y	V	A	P	D	C	R	F	L	T	I	H	R	G	O	V	V	Y	F	S	K	L	-	-	K	G	78		
rat MIA	40	C	S	H	P	I	S	M	A	V	A	L	O	D	Y	V	A	P	D	C	R	F	L	T	I	Y	R	G	O	V	V	Y	F	S	K	L	-	-	K	G	77		
mouse MIA	40	C	S	H	P	I	S	M	A	V	A	L	O	D	Y	V	A	P	D	C	R	F	L	T	I	Y	R	G	O	V	V	Y	F	S	K	L	-	-	K	G	77		
human FDP	77	G	A	G	E	F	W	A	G	S	V	Y	G	D	G	Q	D	E	M	G	V	-	V	G	Y	F	P	R	N	L	V	K	E	R	O	R	V	Y	Q	E	A	T	115
mouse Fdp	77	G	A	G	E	F	W	A	G	S	V	Y	G	D	H	Q	D	E	M	G	I	-	V	G	Y	F	P	S	N	L	V	K	E	R	O	R	V	Y	Q	E	A	T	115
bovin MIA	78	R	G	R	L	F	W	G	G	S	V	O	G	D	Y	Y	G	D	L	A	A	R	L	G	Y	F	P	S	S	I	V	R	E	D	Q	T	L	K	P	A	R	117	
human MIA	79	R	G	R	L	F	W	G	G	S	V	O	G	D	Y	Y	G	D	L	A	A	R	L	G	Y	F	P	S	S	I	V	R	E	D	Q	T	L	K	P	G	K	118	
rat MIA	78	R	G	R	L	F	W	G	G	S	V	O	G	D	Y	Y	G	D	L	A	A	H	L	G	Y	F	P	S	S	I	V	R	E	D	L	T	L	K	P	G	K	117	
mouse MIA	78	R	G	R	L	F	W	G	G	S	V	O	G	D	Y	Y	G	D	L	A	A	R	L	G	Y	F	P	S	S	I	V	R	E	D	L	N	S	K	P	G	K	117	
human FDP	116	K	E	I	P	T	T	D	I	D	F	F	C	E																											128		
mouse Fdp	116	K	E	I	P	T	T	D	I	D	F	F	C	E																										128			
bovin MIA	118	T	D	V	K	T	D	I	W	D	F	Y	C	O																									130				
human MIA	119	V	D	Y	K	T	D	K	W	D	F	Y	C	O																									131				
rat MIA	118	V	D	M	K	T	D	E	W	D	F	Y	C	O																								130					
mouse MIA	118	I	D	M	K	T	D	Q	W	D	F	Y	C	O																								130					

C



probably due to the low expression level of *Fdp*. At E11.5, the periotic mesenchyme has begun to aggregate, condense, and initiate prechondrogenic changes, characterized by the secretion of extracellular matrix components such as sulfated glycosaminoglycans (S-GAG). The chondrogenic program continues between E12 and E13, with a strong increase in S-GAG synthesis. However, at E14, this synthesis decreases in the mesenchymal cells immediately surrounding the otic epithelium, while it remains high in the other areas (21). Consequently, morphological changes appear in the mesenchyme, with the

formation of two concentric rings, the inner ring shows a reduced density compared with the outer ring. (22, 23). From E14 onward, the outermost ring continues its chondrogenesis and differentiates into cartilage, which ultimately gives rise to the bone of the otic capsule. The inner ring cells differentiate into fibrocytes, which have a characteristic shape formed by several cytoplasmic extensions (24, 25). At E17.5, *Fdp* was expressed in the fibrocytes surrounding the cochlear and vestibular duct (Fig. 3A). At around E18, the fibrocytic tissue cavitates to form the scala vestibuli and scala tympani around the cochlear duct,

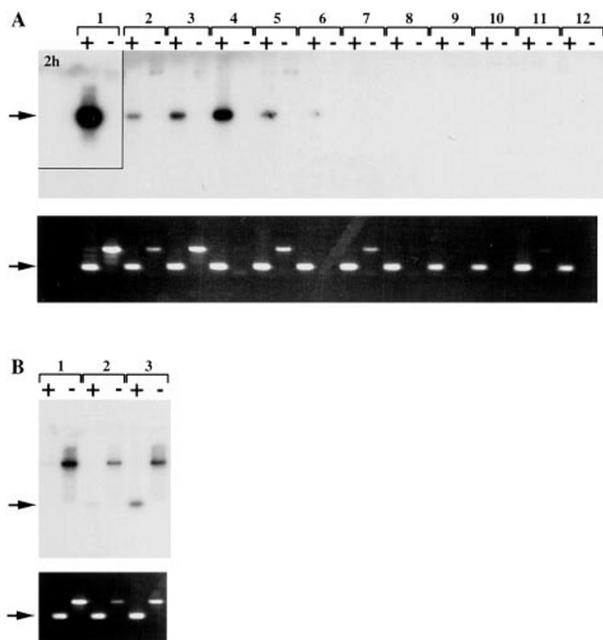


FIG. 2. RT-PCR analysis of *Fdp* expression in mouse tissues. Electrophoresis gel containing the RT-PCR products was transferred and hybridized with a *Fdp* oligonucleotide (see "Experimental Procedures"). **A**, lane 1, blot containing P2 cochlea cDNA was exposed for 2 h at -80°C (the blot containing products from the other RT-PCR experiments was exposed for 24 h at -80°C); lane 2, E10.5 microdissected periotic mesenchyme; lane 3, femoral and clavicle cartilage; lane 4, cranium cartilage; lane 5, eyes; lane 6, brain; lane 7, liver; lane 8, lung; lane 9, skeletal muscle; lane 10, heart; lane 11, intestine; lane 12, testis. **B**, RT-PCR on total embryo RNA: lane 1, E9.5; lane 2, E10.5; lane 3, E11.5. "±" indicates presence or absence of reverse transcriptase in the RT reaction. The arrow in **A** and **B** indicates the 523-bp amplified *Fdp* cDNA band. The higher band of 2.3 kb results from the amplification of genomic DNA. Electrophoresis gel containing the *Hprt* amplification products is presented under each panel. The arrow indicates the 237-bp amplified *Hprt* cDNA band. The higher band of 1 kb results from the amplification of genomic DNA.

which shows a typical snail shape of one and three-quarter turns (22, 23). After birth, in the cochlea, *Fdp* expression was detected in all types of fibrocytes in the spiral ligament behind the stria vascularis, in the suprastrial zone, in the spiral limbus, and in the supralimbal zone, as well as in the mesenchymal cells underlying the basilar membrane (Fig. 3B). No signal was detected in any of the inner ear epithelia or in the cartilage of the otic capsule. The same expression pattern was observed at postnatal day (P) 15 (data not shown).

In Vitro Effect of an *Fdp* Antisense Oligonucleotide on E10.5 Periotic Mesenchyme + Otic Epithelium—The expression pattern of *Fdp* in the E10.5 periotic mesenchyme suggests that this gene could play a role in the early chondrogenic events leading to the formation of the otic capsule. In order to test this hypothesis, we incubated E10.5 mesenchyme + otic epithelium micromass cultures with an *Fdp* antisense oligonucleotide complementary to a region containing the *Fdp* ATG translation start codon (see "Experimental Procedures"). Otocysts with their associated periotic mesenchyme were microdissected and cultured following the micromass technique (26). On culture day 3, sites of mesenchymal condensation, which precede cartilage development, were counted and used as an early indicator of chondrogenesis (13). In untreated cultures and cultures treated with the *Fdp* sense oligonucleotide, numerous mesenchymal condensations formed (Fig. 4; Table I). Condensations of similar size and morphology also developed in *Fdp* antisense oligonucleotide-treated cultures; however, their mean numbers were significantly reduced by 49% (Student's test, $p < 0.05$) in comparison to that of the control cultures (Table I). By culture

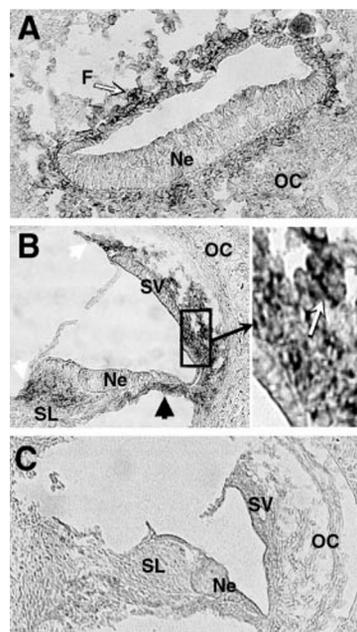


FIG. 3. Inner ear expression of *Fdp* in the cochlea by *in situ* hybridization. **A**, at E17.5, the mesenchyme in the cochlea has differentiated into cartilage, which ultimately gives rise to the bone of the otic capsule (OC), whereas the cells immediately surrounding the neuroepithelium (Ne) differentiate into fibrocytes (F), which express *Fdp*. **B**, at P4 in the cochlea, *Fdp* expression is detected in all types of fibrocytes, in the spiral ligament behind the stria vascularis (SV), in the suprastrial zone (white arrowhead above the stria vascularis), in the spiral limbus (SL), and in the supralimbal zone (white arrowhead above the spiral limbus), as well as in mesenchymal cells underlying the basilar membrane with supports the neuroepithelium (dark arrowhead). The scarred region of the spiral ligament is presented at a higher magnification on the right panel. The white arrow indicates *Fdp* signal in cytoplasm of the fibrocytes. **C**, section of the cochlea at P4 labeled with a *Fdp* sense riboprobe.

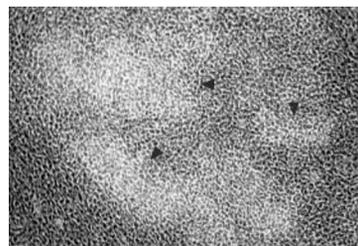


FIG. 4. Culture of E10.5 mouse periotic mesenchyme + otic epithelium. Arrowheads indicate mesenchymal condensations.

day 7, mesenchymal condensations normally differentiate into chondrogenic foci which form the cartilage and synthesize a characteristic extracellular matrix rich in S-GAG. We reasoned that if *Fdp* is essential for chondrogenesis, then the observed effect of blocking *Fdp* with an antisense oligonucleotide on mesenchymal condensation should be reflected in the later chondrogenic event of S-GAG accumulation. We therefore examined the extent of chondrogenesis in response to oligonucleotide treatment by staining the cells with Alcian Blue (pH 1.0), which binds specifically to S-GAG, and quantifying the matrix bound Alcian Blue (see "Experimental Procedures") (27). Mean values for binding of Alcian Blue stain were comparable in untreated cultures and cultures treated with the sense oligonucleotide (Table I). However, in cultures treated with the *Fdp* antisense oligonucleotide, the mean value for binding of Alcian Blue stain was significantly decreased by 48% (Student's test, $p < 0.05$) in comparison to that of the sense oligonucleotide-treated cultures. There is, therefore, a strict correlation between the effect of *Fdp* antisense oligonucleotide treatment on

the number of foci and S-GAG accumulation. In order to test the possible cytotoxic effect of the oligonucleotide treatment, several controls were performed under the same conditions using a random oligonucleotide or an antisense oligonucleotide complementary to the initiation site of the murine β -globin gene. These treatments had no effect on mesenchymal condensation and S-GAG accumulation (Tables I). We could therefore conclude that the observed *Fdp* antisense oligonucleotide effect on chondrogenesis was specific.

Genomic Structure and Chromosomal Mapping of *Fdp* and Its Human Ortholog *FDP*—Based on the role of *Fdp* in the early chondrogenesis of the inner ear, this gene is a good candidate for deafness. We therefore determined the genomic structure of *Fdp* and its human ortholog *FDP* and mapped them to the murine and human genomes, respectively (see “Experimental Procedures”). BAC genomic clones containing the whole *Fdp* and *FDP* genes were isolated and analyzed (see “Experimental Procedures”). As shown in Fig. 1C, *Fdp* contains four exons, which span an approximately 3.2-kb genomic region, as is also the case for *FDP* (data not shown). The *FDP* predicted protein sequence, deduced from the genomic sequence, was 86.7% identical to that of the mouse *Fdp* protein sequence (Fig. 1B).

Part of the human 3'-untranslated region of *FDP* is homologous to sequence-tagged site WI-16380 that maps to 20p11, near the D20S104 and D20S118 markers. The comparative mouse and human map predicted that the murine *Fdp* locus would map to the homologous region on mouse chromosome 2 (Mouse Genome Data Base). The co-segregation of *Fdp* and

chromosome 2 markers in this region was therefore analyzed using an interspecific backcross panel (14) (see “Experimental Procedures”). *Fdp* was mapped between the D2Mit223 and D2Mit22 markers (Fig. 5).

DISCUSSION

Here, we report the isolation of *Fdp*, a novel gene preferentially expressed in the inner ear that encodes a fibrocyte-derived protein. *Fdp* is highly homologous to MIA/CD-RAP (18–20). They both possess a potential signal peptide and exhibit a similar hydrophobicity profile (data not shown) (18). In addition, four cysteine residues, which are thought to form intradisulfide bonds in the human MIA, are also conserved. *Fdp*, like MIA, is therefore likely to be secreted as a small globular protein (18). The *Fdp*-encoding gene also has the same structure as MIA, *i.e.* four exons distributed over a 3.2-kb region. MIA and *Fdp* may thus constitute a new gene family.

MIA/CD-RAP is a cartilage-specific gene. Its expression, which is restricted to chondrocytes and mature cartilage, is correlated with the expression of the chondrocyte-characteristic type IIB splice form of the type II procollagen, and is sensitive to retinoic acid treatment (19, 20). It has been recently reported to be regulated by Sox9, a transcription factor essential for the chondrocyte differentiation and cartilage formation (28, 29). The role of MIA/CD-RAP in cartilage development remains to be clarified. It also has been found to be highly expressed in several malignant human melanoma cell lines, as well as in some neuroectodermally derived tumors, and a Swam rat and human chondrosarcoma (18, 20, 30, 31). In addition to its action as a potent tumor cell growth inhibitor, by blocking DNA replication in human melanoma cell lines (18), MIA/CD-RAP also decreases the adhesive capacity of the cells. Therefore, it may promote tumoral invasion. The relationship between the expression of MIA/CD-RAP in malignant cells and in developing cartilage is still puzzling. Dietz *et al.* (19) have suggested that, as described in tumors, it could control the shape and inhibit division of the chondrocytes that are essential for cartilage differentiation.

Fdp is predominantly expressed in the inner ear, which undergoes chondrogenesis during development. Its expression is first detected in the periotic mesenchyme and then in the developing and mature fibrocytes, which form a loosely aggregated tissue in the perilymphatic spaces between the membranous duct and the cartilage of the otic capsule. The fibrocytes are large cells with several cytoplasmic extensions (24, 25). They have been classified into five different classes, according

TABLE I
Effects of *Fdp* oligonucleotides on chondrogenesis in cultured E10.5 periotic mesenchyme + otic epithelium

Cultures of E10.5 periotic mesenchyme + otic epithelium were grown in the presence or absence of *Fdp* oligonucleotides. Values represent the mean for three to five cultures per experimental group (see “Experimental Procedures”).

Treatment	Condensation no. ^a	S-GAG accumulation ^b
Untreated	75 ± 5	0.789 ± 0.09
β -Globin	80 ± 5	0.823 ± 0.05
<i>Fdp</i> sense	80 ± 5	0.833 ± 0.12
Random	70 ± 5	0.761 ± 0.11
<i>Fdp</i> antisense	37 ± 3 ^c	0.430 ± 0.04 ^c

^a On day 3 of culture, mesenchymal condensations were counted.

^b On day 7 of culture, cells were fixed then stained with Alcian blue 8GX, pH 1.0. Binding of matrix-bound stain was measured spectrophotometrically.

^c Statistically significant at $p < .05$ (Student's test).

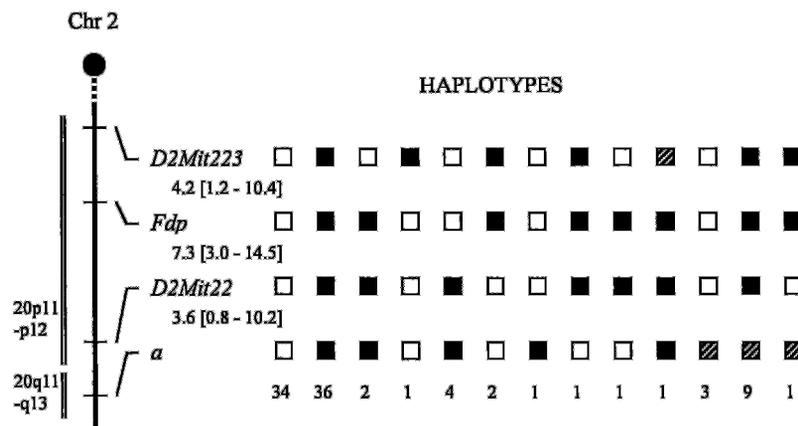


FIG. 5. Genetic mapping of the *Fdp* locus. Line to the left, segment of chromosome 2 with known homology to human chromosome 20 between p11–12 and q11–13. Loci are depicted as horizontal lines along the chromosome, with relative spacing scaled to the distance estimated from our data. Distances with 95% confidence intervals are given between loci. Right, each column represents a haplotype for this set of loci (open squares, homozygotes; black squares, heterozygotes; striped squares, unknown genotypes); the number of mice with a given haplotype is indicated at the bottom of the column.

to their localization, orientation, and ion transport activity (32, 33). These cells, which are connected to each other by gap junctions, have been proposed to play a key role in the regulation of the potassium concentration of the endolymph which bathes the sensory epithelia of the inner ear (34–36). Indeed, the fibrocytes of the spiral ligament, in the lateral wall of the cochlea, are thought to drive the potassium to the stria vascularis, which is responsible for the endolymph secretion (33, 37).

The blocking of *Fdp* expression using antisense oligonucleotides inhibits chondrogenesis in microdissected periotic mesenchyme + otic epithelium in culture. This therefore implies that *Fdp* plays a role in the chondrogenic events that occur during otic capsule development. The controls included in our study demonstrate the specificity of the observed effect; however, the absence of a *Fdp*-specific antibody prevents us from determining whether a low level of *Fdp* expression may exist. The involvement of several growth factors in the early chondrogenesis of the otic capsule has already been demonstrated using similar strategies: fibroblast growth factor 3 (FGF3), using antisense oligonucleotides (38), and transforming growth factor β 1 (39), basic fibroblast growth factor (bFGF) (40), FGF2 and FGF3 (38), and bone morphogenetic protein-2a (41), using purified proteins and their corresponding antibodies. In these studies, as was observed for *Fdp*, the number of condensation foci is affected, rather than their size or morphology, which indicates that these factors play a role in the initiation of chondrogenesis. They are indeed part of a developmental switch, which thereafter leads to the formation of the cartilage of the perilymphatic spaces and to a distinct capsular morphology (13, 21). Gene inactivation approaches have also demonstrated the involvement of other molecules in the formation of the otic capsule, such as the retinoic acid receptors α and γ (42), transforming growth factor β 2 (43), and the transcription factor *Brn4* (37). Interestingly, mutations in this latter gene are responsible for a nonsyndromic form of deafness, linked to the X chromosome (44). The inactivation of *Brn4* results in an abnormal morphology of the otic capsule (37, 45). Based on the coexpression of *Brn4* and *Fdp* in the periotic mesenchyme, we could expect the same phenotype following inactivation of *Fdp* (46).

In addition to the proposed role of *Fdp* in the early morphogenesis of the otic capsule, *Fdp* may later be involved in the differentiation of the fibrocytic tissue *versus* the cartilage, as suggested by its restricted expression in the fibrocytes. It could, as observed *in vitro* for MIA/CD-RAP (18), regulate cell density and shape, which control for fibrocyte differentiation. As discussed above, these cells are involved in the inner ear ion transport mechanisms. Malformation of the fibrocytic tissue, which is observed after inactivation of *Brn4* (37, 45), results in abnormal ionic concentrations of the inner ear fluids, which greatly affect auditory function. The same effect could result from a mutation in *Fdp*.

To date, no locus for deafness has been mapped to the same chromosomal region as *FDP*. However, all loci responsible for deafness are far from being identified, as illustrated by their continuously growing number (47). According to the role of *FDP* in the development of the otic capsule that we demonstrate here using an *in vitro* approach, this gene could be a candidate for deafness associated with malformations of the otic capsule, which comprise approximately 13% of sporadic cases of isolated deafness (48).

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