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Structure and Expression of a Novel Fibroblast Growth Factor, FGF-17, Preferentially Expressed in the Embryonic Brain

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We isolated the cDNA encoding a novel member (216 amino acids) of the fibroblast growth factor (FGF) family from rat embryos. As this protein is the 17th documented member of the FGF family, we tentatively termed it FGF-17. We have also determined the structures of mouse and human FGF-17 with high amino acid identity (100 and 98.6%) to rat FGF-17, respectively. Among FGF family members, FGF-17 is most similar (53.7% amino acid identity) to FGF-8. FGF-17 has a typical signal sequence at its amino terminus. As expected, recombinant rat FGF-17 was efficiently secreted by High Five insect cells infected with recombinant baculovirus containing the cDNA indicating that FGF-17 is a secreted protein. *FGF-17* mRNA of ~2.1 kb was detected in rat embryos at E14.5, but not at E10.5 and E19.5 by Northern analysis. The mRNA was found to be preferentially expressed in the neuroepithelia of the isthmus and septum of the rat embryonic brain at E 14.5 by in situ hybridization. The present results indicate that FGF-17 might be a novel secreted signaling molecule in the induction and patterning of the embryonic brain.

The prototypic fibroblast growth factors (FGFs), FGF-1 (aFGF) and FGF-2 (bFGF), were originally isolated from the brain and pituitary as mitogens for fibroblasts. FGF-1 and FGF-2 are widely expressed in developing and adult tissues, and are polypeptides with multiple biological activities including angiogenesis, mitogenesis, cellular differentiation and repair of tissue injury (1, 2). The FGF family now consists of sixteen members, FGF-1 to FGF-16. They have a conserved ~120-amino acid residue core with ~30 to 60% amino acid identity. FGF-3 was identified to be a common target for activation by the mouse mammary tumor virus (3). FGF-4 to FGF-6 were identified as oncogene products (4-6). FGF-7 to FGF-9 were identified as mitogens for culture cells (7-9). FGF-10 and FGF-16 were identified from the rat lung and heart by homology-based polymerase chain reaction (PCR), respectively (10, 22). FGF-11 to FGF-14 (FGF homologous factors (FHFs)-1 to -4) were identified from the human retina by a combination of random cDNA sequencing, data base searches and homology-based PCR (11). FGF-15 was identified as a downstream target of a chimeric homeodomain oncoprotein (12). These FGFs also appear to play important roles in both developing and adult tissues. Recently, we isolated the cDNA encoding a novel, the 17th documented, member of the FGF family from rat embryos by homology-based PCR. Here, we report the structure and expression of the rat *FGF-17* mRNA.

MATERIALS AND METHODS

Preparation of RNA from rat embryos. RNA was prepared from Wistar rat embryos (embryonic day (E) 10.5, E14.5, and E19.5) or mouse embryos (E13.5) using a RNA extraction kit (Pharmacia Biotech). Poly $(A)^+$RNA was prepared using oligo (dT)-cellulose (Collaborative Research Inc., type 2). The human fetal brain poly $(A)^+$RNA was purchased from Clontech.

Isolation and analysis of the rat, mouse, and human FGF-17 cDNAs. For cDNA synthesis, poly $(A)^+$ RNA was incubated for 60 min at 37 °C in a reaction mixture (20 μl) containing 300 units of Moloney murine leukemia virus reverse transcriptase (Gibco-BRL), 15 units of human placenta RNase inhibitor (Wako Pure Chemicals, Japan) and 0.5 μg of a random hexadeoxynucleotide primer.

The rat *FGF-17* cDNA was amplified from the rat embryo (E14.5)

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cDNA as a template by PCR for 30 cycles at an annealing temperature of 50 °C using sense and antisense degenerate primers representing all possible codons corresponding to the amino acid sequences of mouse FGF-8, ETDTFG and ENNYTA, respectively, (8). DNA of expected size (~150 base pairs) was amplified. The amplified DNA was cloned into the pGEM-T DNA vector (Promega Co.). The nucleotide sequences of the cloned DNAs were determined by a DNA sequencer (Applied Biosystems). To determine the entire coding region, the embryonic cDNA was analyzed by the Rapid Amplification of cDNA Ends (RACE) method (13).

The human *FGF-17* cDNA was also amplified from the human fetal brain cDNA, and analyzed as described above. The mouse *FGF-17* cDNA was amplified from the mouse embryonic cDNA (E13.5) by PCR with primers corresponding to the rat 5′- and 3′-noncoding regions. The apparent evolutionary relationships of members of the FGF family were examined by the UPGMA method with the sequence analysis software, Genetyx (Software Development Co., Tokyo, Japan).

Expression of FGF-17 cDNA in High Five insect cells. The rat *FGF-17* cDNA with a DNA fragment (75 bp) encoding an E tag (GAPVPYPDPLEPR) and a 6X His tag (HHHHHH) at the 3′-terminus of the coding region was constructed in a transfer vector DNA, *pBacPAK9* (Clontech). Recombinant baculovirus containing the *FGF-17* cDNA with the tag sequences was obtained by cotransfection of Sf9 cells with the recombinant *pBacPAK9* and a Bsu36 I-digested expression vector, *BacPAK6* (Clontech). High Five insect cells were infected with the resultant recombinant baculovirus and incubated at 27 °C for 72 h in TC-100 insect medium (Gibco BRL) with 10% fetal bovine serum.

Detection of recombinant FGF-17 by Western blotting analysis. The culture supernatant and cell lysate of High Five cells infected with the recombinant baculovirus were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel (12.5%) electrophoresis under reducing conditions and transferred onto a nitrocellulose membrane (Hybond-ECL, Amersham). The membrane was then incubated at room temperature for 1 h with phosphate-buffered saline (PBS) containing 0.05% Tween 20 and 5% nonfat dry milk. The membrane was incubated at room temperature for 1h with anti-E tag antibodies (1:500) (Pharmacia Biotech) in PBS containing 0.05% Tween 20 and 1% nonfat dry milk. After washing in PBS containing 0.1% Tween 20 and then in PBS, the membrane was treated at room temperature for 1 h with goat anti-mouse immunoglobulin G conjugated to horseradish peroxidase (1:2000) (Cappel). The membrane was washed four times in PBS containing 0.1% Tween 20 and reacted with chemiluminescent horseradish peroxidase substrate (Amersham). The protein with the E tag was visualized with a luminous imaging analyzer (Lumino-CCD, ATTO Co., Japan).

Northern blotting analysis. Aliquots of poly $(A)^+$ RNAs (5 μg) from rat embryos (E10.5, E14.5, and E19.5) were dissolved on a denaturing agarose gel (1%) containing formaldehyde, and transferred to a nitrocellulose membrane in 20× SSC (1× SSC: 0.15 M NaCl/0.015 M sodium citrate) overnight. A ^{32}P-labeled *FGF-17* cDNA probe (~680 bp) was labeled by a random primer labeling kit (Pharmacia Biotech) with deoxycytidine 5′-[α-^{32}P] triphosphate (~110 TBq/mmol) (ICN Biomedicals Inc.). The membrane was then baked at 80 °C for 2 h in a vacuum, and prehybridized at 60 °C for 4 h in hybridization solution (5× SSC/0.1% sodium dodecyl sulfate (SDS)/4× Denhardt's solution/100 μg/ml heat-denatured salmon sperm DNA/5% sodium dextran sulfate), before being hybridized at 60 °C for 18 h in hybridization solution containing the labeled probe. The membrane was then washed at room temperature three times for 20 min each time in 1× SSC and 0.1% SDS, and twice at 60 °C in 0.2× SSC and 0.1% SDS. The washed membrane was analyzed with a radio-imaging analyzer (BAS 2000, Fuji Photo Film Co., Japan).

In situ hybridization. Wistar rat embryos (E14.5) were frozen in powdered dry ice, and sagittal sections were cut at 16 μm with a cryostat, thaw-mounted onto polylysine-coated slides, and stored at −85 °C until hybridization. ^{35}S-labeled rat *FGF-17* antisense and sense cRNA probes were transcribed using SP6 RNA polymerase and T7 RNA polymerase (TaKaRa, Japan) with uridine 5′-α-[^{35}S]-thiotriphosphate (~30 TBq/mmol) (Amersham), respectively. The sections were examined by in situ hybridization with the labeled probe as described (10).

RESULTS AND DISCUSSION

Isolation and analysis of the rat and mouse cDNAs encoding FGF-17. Members of the FGF family have a conserved ~120-amino acid residue core with ~30 to 70% amino acid identity. To isolate cDNAs encoding novel FGFs, cDNAs were amplified from cDNAs of rat adult tissues and embryos as templates by PCR using various sets of primers corresponding to the core of the FGF family and then cloned. We isolated a cDNA fragment encoding a novel FGF from rat embryo cDNA as a template using primers corresponding to amino acid sequences, ETDTFG (amino acids 78 to 83) and ENNYTA (amino acids 124 to 129), of the core of mouse FGF-8 (Fig. 1) (8). The nucleotide sequence of the entire coding region was determined by the RACE method using the rat embryo cDNA as a template. The nucleotide sequence of the coding region allowed elucidation of the complete amino acid sequence of a novel FGF (216 amino acids), which has a conserved amino acid residue core (amino acids 46 to 165) (Fig. 1). As this protein is the 17th documented protein related to FGFs, we tentatively term it FGF-17. We also determined the amino acid sequences of mouse and human FGF-17 which have high amino acid identity (100% and 98.6%) to rat FGF-17, respectively (Fig. 1). Among FGF family members, FGF-17 is most similar (53.7% amino acid identity) to FGF-8 (8) (Fig. 1). The apparent evolutionary relationships of seventeen members of the FGF family are shown in Fig. 2. FGF-17 was closest to FGF-8.

Two cysteine residues are well conserved in the FGF family, and these amino acids correspond to residues 48 and 127 in the FGF-17 sequence (Fig. 1). Although a cysteine residue was found at position 127, a serine instead of a cysteine residue was found at position 48 (Fig. 1). The same substitution was also observed in FGF-8 and FGF-10 sequences (8, 10). Although FGF-1, FGF-9, FHF-1 to FHF-4, and FGF-16 have no typical signal sequence in their termini (2, 11), FGF-3, FGF-4, FGF-5, FGF-6, FGF-7, FGF-9, and FGF-15 have typical signal sequences, and are secreted proteins (3-7, 9, 12). FGF-17 also has a hydrophobic amino terminus (~22 amino acids), which is a typical signal sequence, and appears to be a secreted protein. The signal sequence cleavage site was predicted to lie between amino acid positions 22 (Q) and 23 (T) by the method of von Heijne (14).

Expression of FGF-17 cDNA in High Five insect cells. To produce recombinant FGF-17, High Five insect cells

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Human FGF-17      MGAARLLPNLTLCLQLLILCCQTQGENHPSPNFNQYVRDQGAMTDQLSRRQIREYQLYSR  60
                  ************************************************************
Rat, Mouse FGF-17 MGAARLLPNLTLCLQLLILCCQTQGENHPSPNFNQYVRDQGAMTDQLSRRQIREYQLYSR  60
                                                 * ** *   ******* ** ******
Mouse FGF-8                 MGSPRSALSCLLLHLLVLCLQAQHVREQSLVTDQLSRRLIRTYQLYSR   48

                  TSGKHVQV-TGRRISATAEDGNKFAKLIVETDTFGSRVRIKGAESEKYICMNKRGKLIGK  119
                  ******** ***************************************************
                  TSGKHVQV-TGRRISATAEDGNKFAKLIVETDTFGSRVRIKGAESEKYICMNKRGKLIGK  119
                  ********    ** * ****  ***************  ***   ****** **** *
                  TSGKHVQVLANKRINAMAEDGDPFAKLIVETDTFGSRVRVRGAETGLYICMNKKGKLIAK  108

                  PSGKSKDCVFTEIVLENNYTAFQNARHEGWFMAFTRQGRPRQASRSRQNQREAHFIKRLY  179
                  ************************************************************
                  PSGKSKDCVFTEIVLENNYTAFQNARHEGWFMAFTRQGRPRQASRSRQNQREAHFIKRLY  179
                    ** ***************  ***  *** ***** ****  *  ** *** ** ***
                  SNGKGKDCVFTEIVLENNYTALQNAKYEGWYMAFTRKGRPRKGSKTRQHQREVHFMKRLP  168

                  QGQLPFPNHAEKQKQFEFVGSAPTRRTKRTRRPQPLT                        216
                  *********** ********************  *
                  QGQLPFPNHAERQKQFEFVGSAPTRRTKRTRRPQSQT                        216
                   *       *    ***    *  *  *
                  RGH----HTTEQSLRFEFLNYPPFTRSLRGSQRTWAPEPR                     204
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FIG. 1. Amino acid sequences of rat, mouse, and human FGF-17 and its comparison with mouse FGF-8. Numbers refer to the amino acid positions of FGF-17 and FGF-8. Asterisks indicate identical amino acid residues of the sequences.

were infected with recombinant baculovirus containing the rat *FGF-17* cDNA with the 3′-terminal extension encoding E and 6× His tags. To detect recombinant FGF-17, both the culture supernatant and cell lysate were examined by Western blotting analysis with anti-E tag antibodies. A major band of ~26 kDa was detected only in the culture supernatant (Fig. 3). The observed molecular mass of the major band was essentially consistent with the calculated molecular mass of recombinant FGF-17 (25, 251 Da). This result indicates that FGF-17 is efficiently secreted.

Expression of FGF-17 mRNA in rat embryos. We preliminarily examined the expression of *FGF-17* mRNA in rat embryos (E10.5, E14.5, and E19.5) and adult tissues including the brain, heart, lung, liver, kidney, and small intestine by PCR using specific primers. *FGF-17* mRNA was found to be expressed at significant levels in the embryo at E14.5, and at much lower levels at E10.5 and E19.5, and in all adult tissues

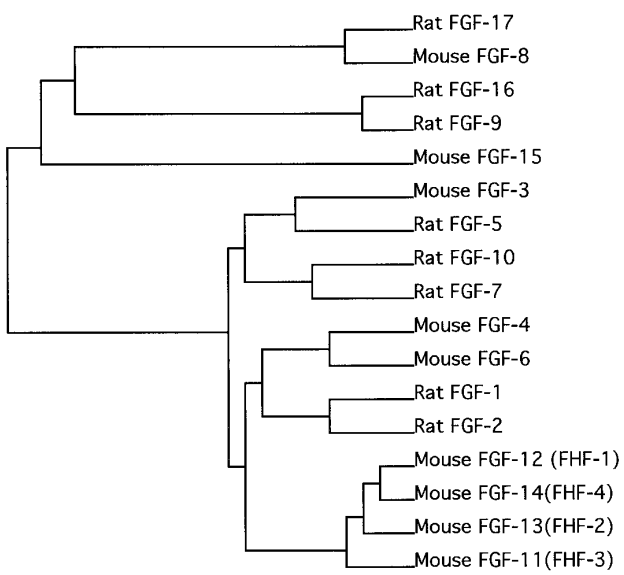

FIG. 2. The apparent evolutionary relationships of 17 members of the FGF family. The length of each horizontal line is proportional to the degree of amino acid sequence divergence.

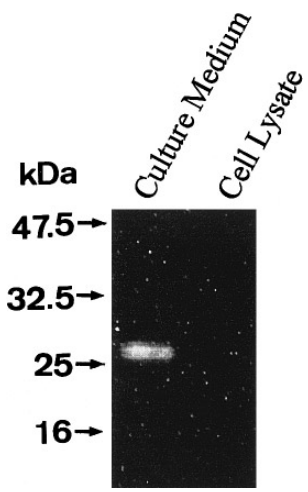

FIG. 3. Detection of recombinant FGF-17 from the culture medium and cell lysate of High Five cells infected with recombinant baculovirus containing the rat *FGF-17* cDNA. The culture medium and cell extracts of the recombinant baculovirus-infected High Five cells were separated by SDS-polyacrylamide gel (12.5%) electrophoresis. Recombinant FGF-17 was detected by Western blotting analysis with anti-E tag antibodies. Prestained Protein Marker Broad Range (New England Biolabs) was used as molecular mass-standard proteins.

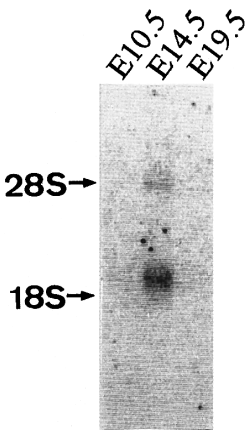

FIG. 4. Expression of *FGF-17* mRNA in rat embryos. Aliquots of poly(A)$^+$ RNA (5 μg each) were electrophoresed on a denaturing agarose gel (1%) containing formaldehyde and transferred onto a nitrocellulose membrane. Hybridization was performed with a ^{32}P-labeled rat *FGF-17* cDNA probe. The positions of 28S and 18S RNAs are indicated as 28S and 18S. Lanes E10.5, E14.5, and E19.5 indicate RNA from rat E10.5, E14.5, and E19.5 embryos, respectively.

examined (data not shown). We also examined the expression of *FGF-17* mRNA in rat embryos by Northern blotting analysis using a ^{32}P-labeled *FGF-17* cDNA probe. The integrity of RNA was confirmed by electrophoresis on a denaturing agarose gel containing formaldehyde (data not shown). The labeled probe mainly hybridized to an mRNA of ~2.1 kb, which is large enough to encode FGF-17, in the embryo at E14.5 (Fig. 3). However, the mRNA was not detected in the embryo at E10.5 and E19.5 (Fig. 4). This result is essentially consistent with that by PCR.

To examine the expression of *FGF-17* mRNA in the embryo at E14.5, sagittal sections of rat embryos (E14.5) were analyzed by in situ hybridization with

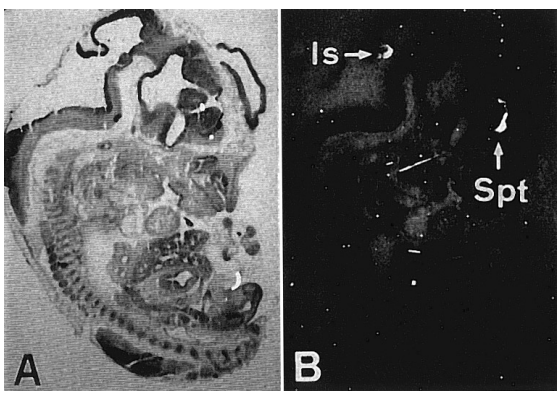

FIG. 5. Localization of *FGF-17* mRNA in a sagittal section of the rat embryo (E14.5). A sagittal section of the embryo was hybridized with an ^{35}S-labeled antisense *FGF-17* cRNA probe and exposed to X-ray film (B). The section was also counterstained with hematoxylin and eosin (A). Is and Spt indicate the isthmus and septum, respectively.

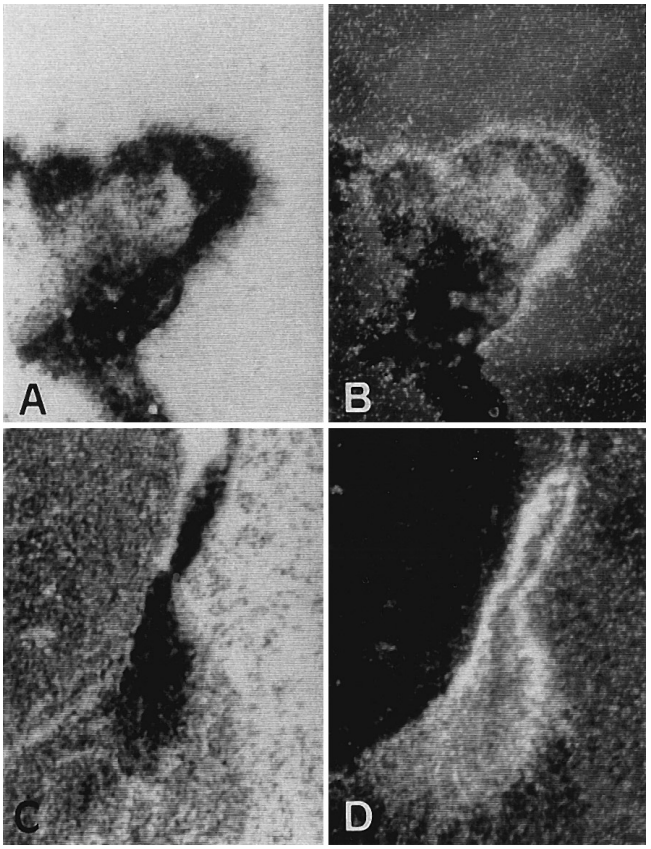

FIG. 6. Localization of *FGF-17* mRNA in the isthmus (A and B) and septum (C and D) of the rat embryonic brain (E14.5). A sagittal section of the embryo was hybridized with an ^{35}S-labeled antisense *FGF-17* cRNA probe and dipped in liquid emulsion. The section was also counterstained with hematoxylin and eosin. Bright-field (A and C) and dark-field (B and D) photomicrographs. B and D, white grains show the localization of *FGF-17* mRNA.

an ^{35}S-labeled antisense or sense *FGF-17* cRNA probe. With the antisense probe, strong discrete labeling was observed preferentially in the brain (Fig. 5). However, no labeling was observed with the sense probe as a control (data not shown), indicating that labeling with the antisense probe was specific for *FGF-17* mRNA. We also examined the expression of *FGF-17* mRNA in the embryo at E19.5 by in situ hybridization. No discrete *FGF-17* mRNA was observed in the embryo at E19.5 (data not shown). This result is essentially consistent with that by Northern blotting analysis. To identify the regions where *FGF-17* mRNA was strongly expressed in the brain, the labeled sections of the embryos were examined by microautoradiography. *FGF-17* mRNA in the embryonic brain was found to be preferentially expressed in the isthmus cerebellar neuroepithelium and septum neuroepithelium (Fig. 6).

FGFs were shown to be expressed in the developing brain. *FGF-3* mRNA was expressed in the mouse developing brain, beginning at E14.5 (20). *FGF-8* mRNA was also expressed in the mouse midbrain-hindbrain junc-

tion (the isthmus) at E9.5 and the mesencephalon-metencephalon at E10.5-12.5 but undetectable at E14.5 (21). *FGF-15* mRNA was also expressed in a regionally restricted pattern in the mouse developing brain (12). In contrast, *FGF-11* to *FGF-14* (*FHF-1* to *FHF-4*) mRNAs were widely expressed in the mouse developing brain (11). However, the expression profile of FGF-17 mRNA was found to be quite different from that of other FGFs.

During developmental processes, FGFs were shown to play important roles in the induction and patterning of neural tissues. FGF-1 and FGF-2 exert differentiation and survival effects on primary cultures from various regions of the embryonic brain (18). FGF-2 can transform anterior neural tissue into neural tissue with a posterior identity (15, 16). FGF signaling is also involved in the target recognition of retinal ganglion cell axons (17). FGF-8 can induce the formation of an ectopic midbrain (19). The present results indicate that FGF-17 might be a novel secreted signaling molecule in the induction and patterning of the embryonic brain.

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