Structure and Expression of the mRNA Encoding a Novel Fibroblast Growth Factor, FGF-18*

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The prototypic fibroblast growth factors (FGFs),¹ FGF-1 (acidic FGF) and FGF-2 (basic FGF), 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 17 members, FGF-1 to FGF-17. They have a conserved amino acid residue core with 120–60% amino acid identity. FGF-18, which was efficiently secreted by High Five insect cells infected with recombinant baculovirus containing the cDNA, induced neurite outgrowth in PC12 cells. The expression of FGF-18 mRNA was examined in adult rat tissues and embryos by Northern blotting analysis and in situ hybridization. FGF-18 mRNA of ~2.7 kilobases was preferentially detected in the lung among adult rat tissues examined. In rat embryos, FGF-18 mRNA was detected in several discrete regions at embryonic days 14.5 and 19.5 but not at E10.5. The temporal and spatial patterns of FGF-18 mRNA expression in embryos are quite different from those of FGF-8 and FGF-17 mRNAs reported. The present results indicate that FGF-18 is a unique secreted signaling molecule in the adult lung and developing tissues.

The mouse mammary tumor virus (3). FGF-4 to FGF-6 were identified as oncogenes (4–6). FGF-7 to FGF-9 were identified as mitogens for culture cells (7–9). FGF-10 was identified from the rat lung by homology-based polymerase chain reaction (PCR) (10). FGF-11 to FGF-14 (FGF homologous factors (FHF)-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). FGF-16 and FGF-17 were identified from the rat heart and embryos by homology-based PCR, respectively (13, 14). These FGFs also appear to play important roles in both developing and adult tissues. Recently, we isolated the cDNA encoding a novel member, the 18th documented, of the FGF family from rat embryos by homology-based PCR. Here, we report the structure and expression of FGF-18 mRNA.

EXPERIMENTAL PROCEDURES

Preparation of RNA—RNA was prepared from adult rat tissues, rat embryos, and mouse embryos using an RNA extraction kit (Amersham Pharmacia Biotech). Poly(A)+ RNA was prepared using oligo(dT)-cellulose (Type 2, Collaborative Biomedical Products, Bedford, MA).

Isolation and Analysis of Rat, Mouse, and Human FGF-18 cDNAs—The cDNA was synthesized from rat embryo (E14.5) poly(A)+ RNA as described (10). To amplify cDNAs, PCR was performed for 30 cycles in 25 µl of a reaction mixture containing an aliquot of the above cDNA solution and 5 pmol/µl of each of the sense and antisense degenerate primers representing all possible codons corresponding to the amino acid sequences, ETDTFG and ENNYTA, of the core of mouse FGF-8 (8), respectively, as described (14). The amplified DNA of expected size (approximately 150 base pairs) was cloned into the pGEM-T DNA vector (Promega, Madison, WI). The nucleotide sequence of the cloned DNA was determined by a DNA sequencer (Applied Biosystems, Foster City, CA). To determine the entire coding region, the rat embryo cDNA was amplified by rapid amplification of cDNA ends (15).

The human FGF-18 cDNA was amplified from the cDNA synthesized from human poly(A)+ RNA (CLONTECH) and analyzed essentially according to the method described above. The mouse FGF-18 cDNA was amplified from the mouse embryo cDNA (E13.5) by PCR with primers corresponding to the rat FGF-18 cDNA sequence and analyzed. The apparent evolutionary relationships of members of the FGF family were examined by the unwaited pair-group method with arithmetic mean method with the sequence analysis software, Genetyx (Software Development Co., Tokyo, Japan).

Production of Recombinant Rat FGF-18 in High Five Insect Cells—The rat FGF-18 cDNA with a DNA fragment (75 base pairs) encoding an E tag (GAPVPPYDPLEPR) and a 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-18 cDNA with the tag sequences was obtained by cotransfection of Sf9 cells with the recombinant pBacPAK9 and a Bsu36I-digested expression vector, BacPAK6 (CLONTECH). High Five insect cells were infected with the resultant recombinant baculovirus and incubated at 27°C for 24 h in TC-100 insect medium (Life Technologies, Inc.) with 10% fetal bovine serum. After the infection, the cells were further...
A Novel FGF, FGF-18

Isolation and Analysis of the Rat, Mouse, and Human cDNAs Encoding FGF-18—Members of the FGF family have a conserved 120-amino acid residue core with 30–70% amino acid identity. To isolate cDNAs encoding novel FGFs, cDNAs were amplified from cDNAs of rat adult tissues and cultured at 27 °C for 60 h in the medium without fetal calf serum.

Detection of Recombinant FGF-18 by Western Blotting Analysis—The culture medium of the cell lysate of FGF-18 cDNA-transfected cells infected with the recombinant baculovirus was separated by SDS-polyacrylamide gel (12.5%) electrophoresis. The recombinant baculovirus was separated by SDS-polyacrylamide gel (12.5%) electrophoresis under reducing conditions and transferred onto a nitrocellulose membrane (Hybond-ECL, Amersham Pharmacia Biotech). The membrane was incubated with anti-E tag antibodies (1:500) (Amersham Pharmacia Biotech). The protein with the E tag was visualized as described (14).

Northern Blotting Analysis—Aliquots of RNAs (20 μg) from rat embryos and adult tissues were dissolved on a denaturing agarose gel (1%)(14) and electrophoresed under reducing conditions and transferred onto a nitrocellulose membrane (Hybond-ECL, Amersham Pharmacia Biotech). The sections were examined by a radio-imaging analyzer (BAS 2000, Fuji Photo Film Co., Tokyo, Japan).

In Situ Hybridization—Wistar rat embryos (E14.5 and E19.5) were frozen in powdered dry ice, and sagittal sections were cut at 16 μm with a cryostat, thaw-mounted onto poly-l-lysine-coated slides, and stored at −85 °C until hybridization. A 35S-labeled FGF-18 cDNA probe (~650 base pairs) was labeled by a random primer labeling kit (Amersham Pharmacia Biotech) with deoxyctydine 5′-α-32P-5′triphosphate (~110 Tbq/mmol) (ICN Biomedicals Inc., Costa Mesa, CA). The membrane was incubated in hybridization solution containing the labeled probe described (14) and analyzed with a radio-imaging analyzer (BAS 2000, Fuji Photo Film Co., Tokyo, Japan).

RESULTS AND DISCUSSION

Asterisks indicate identical amino acid residues of the sequences.

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Northern Blotting Analysis—Aliquots of RNAs (20 μg) from rat embryos and adult tissues were dissolved on a denaturing agarose gel (1%) containing formaldehyde and transferred to a nitrocellulose membrane. The recombinant baculovirus was separated by SDS-polyacrylamide gel (12.5%) electrophoresis under reducing conditions and transferred onto a nitrocellulose membrane (Hybond-ECL, Amersham Pharmacia Biotech). The sections were examined by a radio-imaging analyzer (BAS 2000, Fuji Photo Film Co., Tokyo, Japan).

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RESULTS AND DISCUSSION

Isolation and Analysis of the Rat, Mouse, and Human cDNAs Encoding FGF-18—Members of the FGF family have a conserved 120-amino acid residue core with 30–70% 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 5′ and 3′ ends of the FGF-18 cDNA sequence. The PCR products were separated by agarose gel electrophoresis and purified using a PCR purification kit (Qiagen, Chatsworth, CA). The purified PCR products were cloned into the pGEM-T Easy Vector (Promega, Madison, WI) and sequenced using an automated sequencer (373A, Applied Biosystems, Foster City, CA). The nucleotide and amino acid sequences of the isolated cDNAs were determined using the DNAStar software (DNASTAR, Madison, WI).

Neurite Outgrowth Activity of Recombinant Rat FGF-18 in High Five Insect Cells—To produce recombinant rat FGF-18, High Five insect cells were infected with recombinant baculovirus containing the rat FGF-18 cDNA with the 3′-terminal extension encoding E and His6 tags. To detect recombinant FGF-18, both the culture medium and cell lysate were examined by Western blotting analysis with anti-E tag antibodies. A major band of approximately 28 kDa was detected only in the culture medium, indicating that FGF-18 is efficiently secreted (Fig. 4). The observed molecular mass was larger than the calculated molecular mass of recombinant FGF-18 (23,731 Da). Because a possible N-glycosylation site is found at position 137 (Asn), FGF-18 might be glycosylated.

Neurite Outgrowth Activity of Recombinant Rat FGF-18—FGFs exhibit neurotrophic properties similar to those of neurotrophins (17). The PC12 cell line has provided a useful model for studying the actions of neurotrophins (18). These cells respond to FGFs and neurotrophins by the elaboration of a sym-
pathetic neuron-like phenotype. To examine the biological activity of FGF-18, the culture medium of High Five cells containing FGF-18 was added to PC12 cells. The medium induced neurite outgrowth in PC12 cells (Fig. 5). In contrast, the control medium containing no FGF-18 did not induce neurite outgrowth in PC12 cells. FGF-induced neurite outgrowth in PC12 cells occurs via FGF receptor (FGFR)-1 but not the other FGFRs (FGFR-3 and FGFR-4) expressed in these cells (19).

Expression of FGF-18 mRNA in Adult Rat Tissues and Rat Embryos—The expression of FGF-18 mRNA in adult rat tissues was examined. RNA from the brain, heart, lung, liver, kidney, and small intestine was examined by Northern blotting analysis using a 32P-labeled rat FGF-18 cDNA probe. The positions of 28S and 18S RNAs are indicated. Lanes E10.5, E14.5, E19.5, Brain, Heart, Lung, Liver, Kidney, and Small Intestine indicate RNA from rat E10.5, E14.5, and E19.5 embryos and the adult brain, heart, lung, liver, kidney, and small intestine, respectively.

These results indicate that FGF-18 can activate at least FGFR-1.

Expression of FGF-18 mRNA in Adult Rat Tissues and Rat Embryos—The expression of FGF-18 mRNA in adult rat tissues was examined. RNA from the brain, heart, lung, liver, kidney, and small intestine was examined by Northern blotting analysis using a 32P-labeled FGF-18 cDNA probe. The integrity of RNA was confirmed by electrophoresis on a denaturing agarose gel containing formaldehyde. The labeled probe hybridized to a mRNA of approximately 2.7 kilobases in the lung (Fig. 6). However, the mRNA was not detected in the brain, heart, liver, kidney, and small intestine. The amino acid sequence of FGF-18 is highly homologous to that of FGF-8 and FGF-17. Among adult tissues, the FGF-8 mRNA expression was weak and confined to the gonadal tissue (20). FGF-17 mRNA was not detected in adult major tissues examined (14). In contrast, FGF-18 mRNA was abundantly expressed in the lung. The expression profile of FGF-18 mRNA is quite different from that of FGF-8 and FGF-17 mRNAs.
To examine the expression of FGF-18 mRNA in rat embryos, RNA from embryos at three different embryonic stages (E10.5, E14.5, and E19.5) was also examined by Northern blotting analysis. FGF-18 mRNA was detected in the embryos at E14.5 and E19.5, but not at E10.5 (Fig. 6). The expression of FGF-18 mRNA in the embryos at E14.5 and E19.5 was also examined by *in situ* hybridization with a $^{35}$S-labeled antisense FGF-18 cRNA probe, followed by macroautoradiography. In the embryo at E14.5, discrete labeling was observed in various regions including the isthmus, pituitary, spinal cord, tongue, intervertebral disc, dorsal root ganglion, and pelvis (Fig. 7). In contrast, discrete labeling in the embryo at E19.5 was observed in restricted regions including the lung and anterior pituitary (Fig. 7).

FGF-8 mRNA was detected at E10.5 to E12.5 but not at E13.5 in mouse embryos (20). FGF-17 mRNA was detected at E14.5 but not at E10.5 and E19.5 in rat embryos (14). In contrast, FGF-18 mRNA was detected at E14.5 and E19.5, but not at E10.5 in rat embryos. The spatial expression patterns of FGF-8 and FGF-17 mRNAs in embryos were highly restricted (14, 20). However, FGF-18 mRNA was expressed in various regions in rat embryos. The temporal and spatial patterns of FGF-18 mRNA expression in embryos is also quite different from those of FGF-8 and FGF-17 mRNAs.

During developmental processes, FGFs were shown to play important roles in the induction and patterning of developing tissues. FGF-8 was shown to be an important signaling molecule in the midbrain development and limb development (21, 22). FGF-17 is also thought to be a signaling molecule in the midbrain and forebrain (14). The present results indicate that FGF-18 is a unique secreted signaling molecule in the developing tissues.

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