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Structure and Expression of a Novel Member, FGF-16, of the Fibroblast Growth Factor Family

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We have isolated cDNA encoding a novel member (207 amino acids) of the FGF family from the rat heart by homology-based polymerase chain reaction. As this protein is the 16th documented member of the FGF family, we tentatively term it FGF-16. Among FGF family members, FGF-16 is most similar (73% amino acid identity) to FGF-9. We have also determined the structure of human FGF-16 with high amino acid sequence identity (98.6%) to rat FGF-16. Although the predicted FGF-16 amino acid sequence lacks a typical signal sequence, recombinant rat FGF-16 was efficiently secreted by Sf9 insect cells infected with recombinant baculovirus containing the cDNA. *FGF-16* mRNA was predominantly expressed in the rat heart among the adult major tissues examined. The expression profile of *FGF-16* mRNA was quite different from those of other members of the FGF family. In rat embryos, *FGF-16* mRNA was predominantly expressed in the brown adipose tissue. However, the expression decreased greatly after birth. These results indicate that FGF-16 in embryos might play a role in development of the brown adipose tissue.

The prototypic fibroblast growth factors (FGFs), FGF-1 (aFGF) and FGF-2 (bFGF), originally isolated as mitogens for fibroblasts from the brain and pituitary, 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 fifteen members (1-11, 18). These FGFs also appear to play important roles as peptide growth factors in both developing and adult tissues. FGF-3 (int-2), FGF-4 (hst/kFGF), FGF-5 and FGF-6 were identified as oncogene products (5-8). FGF-7 (KGF) was isolated as a mitogen for cultured keratinocytes (9). FGF-8 and FGF-9 were isolated as an androgen-induced growth factor and a glia-activating factor from mouse mammary carcinoma cells and human glioma cells, respectively (3, 4). FGF-10 was identified from rat embryos by homology-based polymerase chain reaction (PCR) (10). These *FGFs* are predominantly expressed during embryonic development and in restricted adult tissues. Furthermore, four novel members, FGF homologous factors (FHFs), FHF-1 to FHF-4, were identified from the human retina by a combination of random cDNA sequencing, data base searches and homology-based PCR (11). *FHFs* are predominantly expressed in the developing and adult nervous system. Coulier et al. proposed that FHF-1, FHF-2, FHF-3, and FHF-4 should be designated FGF-12, FGF-13, FGF-11, and FGF-14 (16, 17). The 15th documented member of the family, FGF-15, was also identified as a downstream target of the chimeric homeodomain oncoprotein E2A-Pbx1 (18).

FGFs have a conserved ~120-amino acid residue core with ~30 to 70% amino acid sequence identity (1-11). We have tried to isolate the cDNAs encoding novel FGFs by homology-based PCR with primers specific for conserved amino acid sequences within the FGF family. Recently, we isolated the cDNA encoding a novel, the 16th documented, member of the FGF family from the heart. Here, we report the structures and expression of the rat and human mRNAs encoding the novel FGF.

MATERIALS AND METHODS

Preparation of RNA from rat embryos and adult tissues. RNA was prepared from Wistar rat tissues using an RNA extraction kit

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The nucleotide sequence data reported in this paper will appear in the DDBJ, EMBL and GenBank Nucleotide Sequence Databases with the following accession numbers AB002561 and AB009391.

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(Pharmacia Biotech). Poly $(A)^+$ RNA was prepared using oligo(dT)-cellulose (Collaborative Research Inc., type 2).

Isolation and analysis of the rat and human FGF-16 cDNAs. *FGF* family cDNAs was amplified from cDNA synthesized from rat brain poly $(A)^+$ RNA by PCR with 5 pmole/μl of each of the sense and antisense degenerate primers representing all possible codons corresponding to the consensus amino acid sequences of mouse FGF-3 and FGF-7, YLAMNK and YNTYAS, respectively (12, 13). The amplified DNA of expected size (~110 base pairs) was cloned into the pGEM-T DNA vector (Promega Co.). The nucleotide sequence of the cloned DNA was determined by a DNA sequencer (Applied Biosystems). To determine the entire coding region, cDNA synthesized from rat heart poly $(A)^+$ RNA was analyzed by the Rapid Amplification of cDNA Ends (RACE) method (14). 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).

The human *FGF-16* gene fragments were amplified from the genomic DNA by PCR with pairs of primers designed from the rat FGF-16 sequence. The sequence of one short amplification product was used to design human specific *FGF-16* primers. To determine the entire coding region, cDNA synthesized from human heart poly $(A)^+$ RNA (Clontech) and the genomic DNA were analyzed by the RACE method (14).

Expression of FGF-16 cDNA in Sf9 insect cells. An *FGF-16* 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-16* 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). Sf9 insect cells were infected with the resultant recombinant baculovirus and incubated at 27 °C for 24 h in TC-100 insect medium (Gibco BRL) with 10% fetal bovine serum. After incubation, Sf9 cells were washed with TC-100 without fetal bovine serum and further incubated to produce recombinant FGF-16 at 27 °C for 36 h in TC-100 without fetal bovine serum.

Detection of recombinant FGF-16 by Western blotting analysis. The culture supernatant and cell lysate of Sf9 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 1% nonfat dry milk, 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 chemiluninescent horseradish peroxidase substrate (Amersham). The protein with the E tag was visualized by exposure of the membrane to X-ray film (RX Medical, Fuji Film Co., Japan).

Northern blotting analysis. Aliquots of RNA (10 μg) from rat tissues 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. 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 a ^{32}P-labeled *FGF -16* cDNA probe labeled by a random primer labeling kit (TaKaRa, Japan) with deoxycytidine 5′-[α-^{32}P] triphosphate (~110 TBq/mmol) (ICN Biomedicals Inc.). 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 radioimaging analyzer (BAS 2000, Fuji Photo Film Co., Japan).

In situ hybridization. Wistar rat embryos (embryonic day 19.5, E19.5) were frozen in powdered dry ice, and sagittal sections were cut at 16 μm by a cryostat, thaw-mounted onto polylysine-coated slides, and stored at −85 °C until hybridization. ^{35}S-labeled rat *FGF-16* 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). To determine the regional localization of the rat FGF mRNA, labeled sections were exposed to X ray film (Hyperfilm-β max, Amersham). The sections were visualized by counterstaining with hematoxylin and eosin.

RESULTS AND DISCUSSION

Isolation and analysis of the rat and human FGF-16 cDNAs. Members of the FGF family have a conserved ~-120-amino acid residue core with ~30 to 70% amino acid sequence identity. Amino acids 96 to 101 (YLAMNK) and 126 to 131 (YNTYAS) of FGF-3 are identical with those of the corresponding regions of FGF-7 (12, 13). Thus, we designed degenerate oligonucleotide primers representing all possible codons corresponding to the consensus sequences of FGF-3 and FGF-7 to isolate cDNA fragments encoding novel members of the FGF family by polymerase chain reaction (PCR). We tried to isolate cDNA fragments encoding novel members of the FGF family from the rat brain by PCR using the consensus primers described above. DNA of expected size (~110 base pairs), which was a major amplified product (data not shown), was cloned. Fifty-five clones were isolated, and their nucleotide sequences were determined. Forty-seven clones were found to be FGF-related cDNA clones. Among them, only one clone had a sequence which was similar to but distinct from the cDNAs encoding known members of the FGF family, suggesting that the cDNA encodes a novel member of the FGF family.

The expression of the mRNA encoding the novel FGF in adult rat tissues was preliminarily examined by PCR with primers specific for the mRNA. The heart was found to express the mRNA much more abundantly than the brain (data not shown). Next, the cDNA covering the entire coding region of the FGF was isolated from the heart by the Rapid Amplification of cDNA Ends (RACE) method (14). The nucleotide sequence of the coding region of the cDNA allowed elucidation of the complete amino acid sequence of a novel FGF (207 amino acids), which has a conserved ~120-amino acid residue core (amino acids 58–149 and 161–189) (Fig. 1). The cDNA fragment was originally amplified by homology-based PCR with primers for the consensus amino acid sequences of FGF-3 and FGF-7. Although one consensus sequence, YNTYAS, was found at amino acids 144 to 149 of the protein, another consensus se-

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Human FGF-16 MA---EVGGVFASLDWDLHGFSSSLGNVP-LA-DSPGFLNERLGQIE-GKLQRGSP-TDFAH  55
             **   ************* ********** ** ************** ******** *****
  Rat FGF-16 MA---EVGGVFASLDWDLQGFSSSLGNVP-LA-DSPGFLNERLGQIE-GKLQRGSP-TDFAH  55
             **   ***  *   *          **** *  ***  *   *** * * * **   **  *
  Rat FGF-9  MAPLGEVGSYFGVQDAVPF------GNVPVLPVDSPVLLSDHLGQSEAGGLPRGPAVTDLDH  56

             LKGILRRRQLYCRTGFHLEIFPNGTVHGTRHDHSRFGILEFISLAVGLISIRGVDSGLYL 115
             ************************************************************
             LKGILRRRQLYCRTGFHLEIFPNGTVHGTRHDHSRFGILEFISLAVGLISIRGVDSGLYL 115
             ************************  *** ************ **** ************
             LKGILRRRQLYCRTGFHLEIFPNGTIQGTRKDHSRFGILEFISIAVGLVSIRGVDSGLYL 116

             GMNERGELYGSKKLTRECVFREQFEENWYNTYASTLYKHSDSERQYYVALNKDGSPREGY 175
             ******** ***************************************************
             GMNERGELFGSKKLTRECVFREQFEENWYNTYASTLYKHSDSERQYYVALNKDGSPREGY 175
             **** *** ** *** ********************* * **** *  * ********* ****
             GMNEKGELYGSEKLTQECVFREQFEENWYNTYSSNLYKHVDTGRRYYVALNKDGTPREGT 176

             RTKRHQKFTHFLPRPVDPSKLPSMSRDLFHYR 207
             ***************************** **
             RTKRHQKFTHFLPRPVDPSKLPSMSRDLFRYR 207
             ****************** * *    *
             RTKRHQKFTHFLPRPVDPDKVPELYKDILSQS 208
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FIG. 1. Amino acid sequence comparison of rat FGF-16 with human FGF-16 and rat FGF-9. Numbers refer to the amino acid positions of rat FGF-16, human FGF-16 and rat FGF-9. Asterisks indicate identical amino acid residues of the sequences.

quence, YLAMNK, was not found in the amino acid sequence (Fig. 1). However, a sequence similar to the latter consensus sequence was found at amino acids 114 to 119, YLGMNE (Fig. 1). This result indicates that the cDNA fragment was amplified from the brain cDNA by PCR with a mismatched primer.

Two cysteine residues that are well conserved in the FGF family are also conserved in the protein (amino acids 67 and 133). As this protein is the 16th documented member of the FGF family, we tentatively term it FGF-16. The amino acid sequence of FGF-16 was found to be most homologous (73%) to that of FGF-9 (Fig. 1). The apparent evolutionary relationships of sixteen members of the FGF family are shown in Fig. 2. FGF-16 was closest to FGF-9. The human *FGF-16* cDNA fragments covering the entire coding region was amplified from the genomic DNA and heart cDNA by PCR. Determination of their nucleotide sequences allowed elucidation of the complete amino acid sequence of human FGF-16 (207 amino acids) with high sequence identity (98.6%) to rat FGF-16 (Fig. 1).

Expression of FGF-16 cDNA in Sf9 insect cells. FGF-3, FGF-4, FGF-5, FGF-6, FGF-7, and FGF-8 with typical signal sequences at their amino termini are efficiently secreted from cells (3, 5-8). In contrast, FGF-1, FGF-2, FGF-9 and FHF-1 to FHF-4 have no typical signal sequence at their amino termini (1, 2, 4, 11).

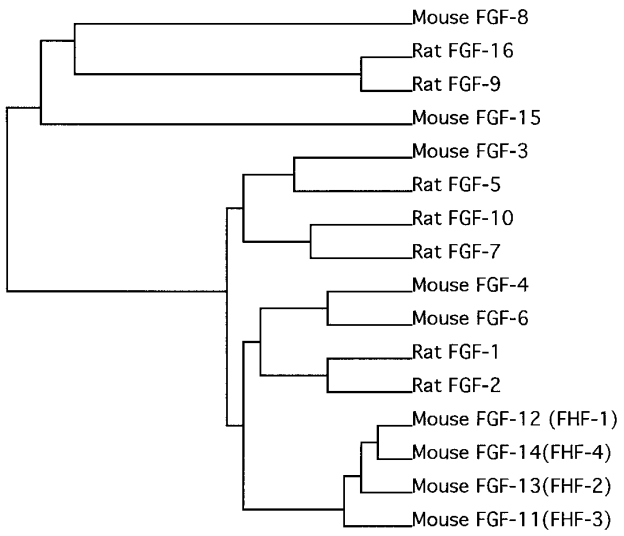

FIG. 2. The apparent evolutionary relationships of sixteen members of the FGF family. The length of each horizontal line is proportional to the degree of amino acid sequence divergence.

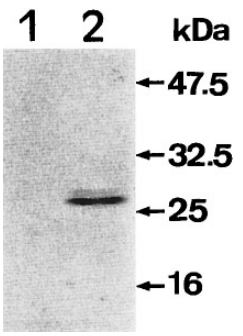

FIG. 3. Detection of recombinant FGF-16 from the culture medium and cell lysate of Sf9 cells infected with recombinant baculovirus containing the rat *FGF-16* cDNA. The culture medium and cell extracts of the recombinant baculovirus-infected Sf9 cells were separated by SDS-polyacrylamide gel (12.5%) electrophoresis. Recombinant FGF-16 was detected by Western blotting analysis with anti-E tag antibodies. Lane 1, cell lysate; lane 2, culture medium. Prestained Protein Marker Broad Range (New England Biolabs) was used as molecular mass-standard proteins.

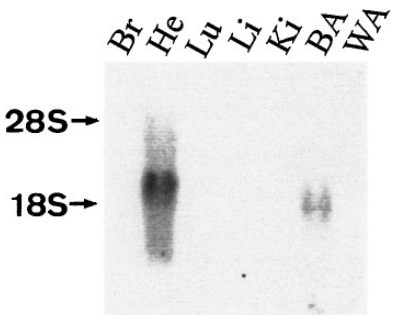

FIG. 4. Expression of *FGF-16* mRNA in the adult tissues. Aliquots of RNA (10 μg each) were electrophoresed on a denaturing agarose gel (1%) containing formaldehyde, and were transferred onto a nitrocellulose membrane. Hybridization was performed with a ^{32}P-labeled rat *FGF-16* cDNA probe. The positions of 28S and 18S RNAs are indicated as 28S and 18S. Lanes Br, He, Lu, Li, Ki, BA and WA indicate RNA from the adult brain, heart, lung, liver, kidney, brown adipose tissue and white adipose tissue, respectively.

FGF-1, FGF-2 and FHF-1 are not secreted. In contrast, FGF-9 is efficiently secreted. Hydropathy plot analysis (15) showed the low value of the amino-terminal region of FGF-16, indicating that FGF-16 has no typical signal sequence (data not shown). To examine if FGF-16 is secreted, Sf9 insect cells were infected with recombinant baculovirus containing the rat *FGF-16* cDNA with the 3′-terminal extension encoding E and 6X His tags. To detect recombinant FGF-16 with carboxy-terminal 25-amino acid extension of the tags, 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 mainly in the culture supernatant (Fig. 3). The observed molecular mass of the major band was consistent with the calculated molecular mass of recombinant FGF-16 (26,462 Da). This result indicates that FGF-16 is also efficiently secreted.

Expression of FGF-16 mRNA in adult rat tissues. We examined the expression of *FGF-16* mRNA in adult rat tissues. RNA from the brain, heart, lung, liver, kidney, brown adipose tissue and white adipose tissue was examined by Northern blotting analysis using a ^{32}P-labeled *FGF-16* cDNA probe. The integrity of RNA was confirmed by electrophoresis on a denaturing agarose gel containing formaldehyde (data not shown). The labeled probe strongly hybridized to a mRNA of 1.8 kb in the heart (Fig. 4). The labeled mRNA was also weakly detected in the brown adipose tissue. However, the mRNA was not detected in the brain, lung, liver, kidney and white adipose tissue. We also examined the expression of *FGF-16* mRNA in other tissues including the small intestine, muscle, thymus, stomach, pancreas, spleen and testis by PCR with specific primers for rat *FGF-16* mRNA. *FGF-16* mRNA was detected in these tissues at much lower levels than in the heart (data not shown). Thus, *FGF-16* mRNA is predominantly expressed in the heart. *FGF-9* mRNA is predominantly expressed in the kidney (4). Although the amino acid sequence of FGF-16 is highly homologous to that of FGF-9, the expression profile of *FGF-16* mRNA is quite different from that of *FGF-9* mRNA.

To examine the expression of *FGF-16* mRNA in the rat heart, sagittal sections of the heart were analyzed by in situ hybridization with a ^{35}S-labeled antisense *FGF-16* cRNA probe. Diffuse labeling was observed in the heart (unpublished observation). This result indicates that *FGF-16* mRNA is expressed in cardiac myocytes. To examine the biological activity of FGF-16, mice were administered recombinant rat FGF-16 produced in E. coli. via intraperitoneal injection. However, no biological effect of FGF-16 on the heart was observed. FGF-16 induced only hepatocellular proliferation (unpublished observation). Although the physiological role of FGF-16 in adults remains to be elucidated, FGF-16 is expected to play unique roles.

Expression of FGF-16 mRNA in rat embryos. We examined the expression of *FGF-16* mRNA in rat em-

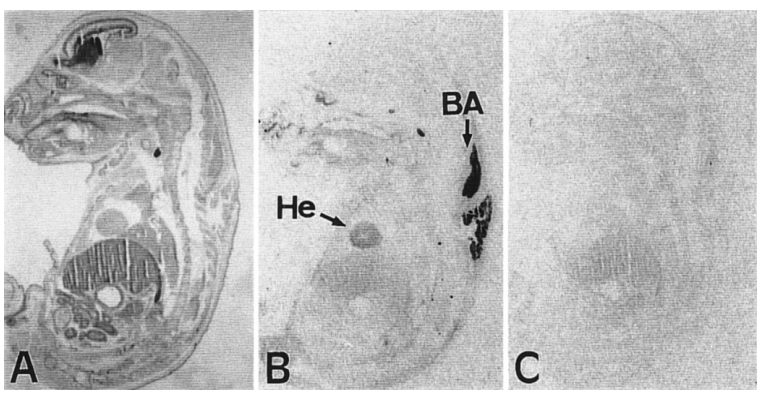

FIG. 5. Localization of *FGF-16* mRNA in a sagittal section of the rat embryo (E19.5). A sagittal section of the embryo was hybridized with a ^{35}S-labeled antisense (B) or sense (C) *FGF-16* cRNA probe. The section was also counterstained with hemaoxylin and eosin (A). He and BA indicate the heart and brown adipose tissue, respectively.

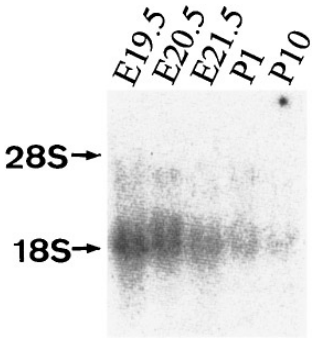

FIG. 6. Expression of *FGF-16* mRNA in the brown adipose tissue at different developmental stages. Aliquots of RNA (10 μg each) were electrophoresed on a denaturing agarose gel (1%) containing formaldehyde, and were transferred onto a nitrocellulose membrane. Hybridization was performed with a ^{32}P-labeled rat *FGF-16* cDNA probe. The positions of 28S and 18S RNAs are indicated as 28S and 18S. Lanes E19.5, E20.5, E21.5, P1 and P10 indicate RNA from the brown adipose tissue at E19.5, E20.5, E21.5, P1 and P10, respectively.

bryos (E10.5, E14.5 and E19.5) by PCR. *FGF-16* mRNA was found to be significantly expressed at E19.5, but at much lower levels at E10.5 and E14.5 (data not shown). To examine the expression of FGF-16 mRNA in the embryo at E19.5, sagittal sections of embryos (E19.5) were analyzed by in situ hybridization with a ^{35}S-labeled antisense or sense *FGF-16* cRNA probe, followed by macroautoradiography. With the antisense probe, strong discrete labeling was preferentially observed in the brown adipose tissue. In the heart, only weak labeling was observed (Fig. 5B). The brown adipose tissue is a major site for lipid metabolism specialized in the non-shivering thermogenesis required to address the physiological hypothermia, in newborn mammals. Development of the brown adipose tissue in the rat occurs mainly during the perinatal period (19). We examined the expression of *FGF-16* mRNA in the brown adipose tissue at different developmental stages (E19.5 to postnatal day 10, P10) by Northern blotting analysis (Fig. 6). The expression of *FGF-16* mRNA in the brown adipose tissue was found to decrease greatly after birth. These results indicate that FGF-16 in embryos might play an role in development of the brown adipose tissue.

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