Fibroblast Growth Factor-16 Is a Growth Factor for Embryonic Brown Adipocytes*

(Moricohika Konishi, Tadahisa Mikami, Masahiro Yamasaki, Ayumi Miyake, and Nobuyuki Itoh‡

From the Department of Genetic Biochemistry, Kyoto University Graduate School of Pharmaceutical Sciences, Sakyo, Kyoto 606-8501, Japan

In rat embryos, fibroblast growth factor (FGF)-16 is predominantly expressed in brown adipose tissue. To elucidate the role of FGF-16, we examined the expression of FGF-16 mRNA in rat embryonic brown adipose tissue at different developmental stages by Northern blotting analysis and in situ hybridization. FGF-16 mRNA was expressed abundantly in brown adipose tissue during embryonic day 17.5, embryonic days 17.5–19.5, and thereafter at lower levels into the neonatal period. The expression profile of FGF-16 mRNA well corresponds to the proliferative profile of embryonic brown adipose tissue reported. We also examined the mitogenic activity of recombinant rat FGF-16 for primary brown adipocytes prepared from rat embryonic brown adipose tissue. FGF-16 showed significant mitogenic activity for primary brown adipocytes. The mitogenic activity was found to be exerted by binding and activating FGF receptor-4 in the brown adipose tissue. As a great induction of proliferation of rat brown adipose tissue during cold acclimation was reported, we also examined the expression of FGF-16 mRNA in the brown adipose tissue during cold acclimation by Northern blotting analysis. The expression of FGF-16 mRNA was not increased, but rather decreased. The expression profile of FGF-16 mRNA and the mitogenic activity of FGF-16 reported here indicate that FGF-16 is a unique growth factor involved in proliferation of embryonic brown adipose tissue.

Adipose tissues serve an important function in the energy economy of vertebrate organisms by providing a massive energy reserve that can be mobilized upon demand. There are two types of adipose tissues, white and brown. Their physiological roles are quite different. White adipose tissue stores energy, whereas brown adipose tissue dissipates energy. Brown adipose tissue is a specialized tissue of mammals responsible for facultative thermogenesis. Its physiological significance has been recognized in newborns when the decrease in environmental temperature at birth requires an adaptive increase in heat production (1). Brown adipose tissue is also speculated to normally function to prevent obesity. Indeed, transgenic mice with primary deficiency of brown adipose tissue have obesity, which develops in the absence of hyperphagia. As obesity progresses, transgenic animals develop hyperphagia (2). These results support a critical role for brown adipose tissue in the nutritional homeostasis of mice.

The prototypic fibroblast growth factors (FGFs),† FGF-1 (acidic FGF) and FGF-2 (basic FGF), were originally isolated from the brain and pituitary as mitogens for fibroblasts (3, 4). The FGF family now consists of 19 members, FGF-1 to FGF-19 (5). FGFs are widely expressed in developing and adult tissues and appear to have important roles with multiple biological activities, including angiogenesis, mitogenesis, cellular differentiation, and repair of tissue injury (3, 4, 7, 8). In rat embryos, FGF-16 mRNA was predominantly expressed in brown adipose tissue, indicating that FGF-16 plays a unique role in embryonic brown adipose tissue (9). In the present study, we examined the expression of FGF-16 mRNA in rat brown adipose tissue at different developmental stages in embryos and during cold acclimation in adults and the mitogenic activity of FGF-16 for primary rat brown adipocytes. The present findings indicate that FGF-16 is a unique growth factor involved in proliferation of embryonic brown adipose tissue.

EXPERIMENTAL PROCEDURES

Preparation of RNA—RNA was prepared from Wistar rat brown adipose tissue using an RNA extraction kit (Amersham Pharmacia Biotech).

Northern Blotting Analysis—Aliquots of RNA 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 32P-labeled rat FGF-16 cDNA (9) probe or UCP-1 cDNA (13) probe was labeled with a random primer labeling kit (Amersham Pharmacia Biotech) with deoxycytidine 5′-α-32P-triphosphate (~110 TBq/mmol) (ICN Biochemicals Inc.). The membrane was incubated in hybridization solution containing the labeled probe as described (9) and analyzed with a radioimaging analyzer (Bas 2000, Fuji Photo Film Co., Tokyo, Japan).

In Situ Hybridization—Wistar rat embryos were frozen in powdered dry ice, and sagittal sections of interscapular areas were cut at 16 μm with a cryostat, thaw-mounted onto polylysine-coated slides, and stored at −85 °C until hybridization. An 35S-labeled rat FGF-16 antisense cRNA probe was transcribed using SP6 RNA polymerase (TaKaRa, Japan) with uridine 5′-α-35S-thiotriphosphate (~30 TBq/mmol) (Amersham Pharmacia Biotech). 32S-Labeled rat FGF receptor antisense cRNA probes were prepared as described (17). The sections were examined in situ hybridization with the labeled antisense probe as described (9) and exposed to x-ray film (Hyperfilm-β max, Amersham Pharmacia Biotech) for 10 days. The labeled sections were also dipped in liquid emulsion (Kodak NTB3) diluted 1:1 with water. After 4 weeks of exposure, they were developed with D-19 (Eastman Kodak Co.) and counterstained with hematoxylin and eosin.

Preparation of Primary Rat Embryonic Brown Adipocytes—Interscapular brown adipose tissue was dissected out from rat embryos at embryonic day 21.5. Primary brown adipocytes were prepared from the

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‡ To whom correspondence should be addressed: Dept. of Genetic Biochemistry, Kyoto University Graduate School of Pharmaceutical Sciences, Yoshida-Shinomachi, Sakyo, Kyoto 606-8501, Japan. Tel.: 81-75-753-4540; Fax: 81-75-753-4600; E-mail: itohnobu@pharm.kyoto-u.ac.jp.

† The abbreviations used are: FGF, fibroblast growth factor; FGFR, FGF receptor.

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brown adipose tissue essentially according to the method of Bronnikov et al. (15). The brown adipose tissue was incubated in Hanks’ solution (pH 7.4) containing 0.2% crude collagenase type I (Sigma) for 1 h at 37 °C. The cell suspension was cooled on ice for 15 min, and the infranatant was filtered through a 200-μm nylon screen and centrifuged for 2 min at 700 × g. The packed cells were washed with medium 199 and suspended in modified Eagle’s medium supplemented with 10% fetal calf serum (Life Technologies, Inc.).

Mitogenic Activity Assay—Recombinant rat FGF-16 was prepared in *Escherichia coli* as described (12). Mitogenic activity of recombinant FGF-16 for primary rat embryonic brown adipocytes cultured in a serum-free medium was determined essentially according to the method of Teruel et al. (20). Primary rat embryonic brown adipocytes were plated onto 24-well plates (2 × 10^4 cells/well) and cultured in modified Eagle’s medium with 10% fetal calf serum. After 24 h in culture at 37 °C in an atmosphere of 5% CO_2_, the cells were washed twice with phosphate-buffered saline (PBS) and were cultured in modified Eagle’s medium with 0.2% bovine serum albumin for 20 h at 37 °C. The cells were further cultured in modified Eagle’s medium with 0.2% bovine serum albumin and 0–25 ng/ml recombinant rat FGF-16. After 20 h, [methyl-^3^H]thymidine (740 GBq/mmol, 7.4 Kibq/ml) (NEN Life Science Products) was added to the culture medium, and the cells were further cultured for 4 h. [^3^H]Thymidine incorporation into acid-insoluble material was determined by a liquid scintillation counter.

Identification of Alternatively Spliced Variants of FGF Receptors 1 and 2 Expressed in Rat Embryonic Brown Adipose Tissue—The cDNAs encoding extracellular immunoglobulin-like domain III with surrounding regions of FGF receptors 1 and 2 (FGFR-1 and FGFR-2) were amplified from rat embryonic brown adipose tissue cDNA (embryonic day 19.5) by polymerase chain reaction. Alternatively spliced variants were identified by determination of the nucleotide sequence of the FGF-1 cDNA and enzymatic digestion of the FGF-2 cDNA with *Hae* III as described (18).

Preparation of Recombinant Extracellular Domains of FGFR-1c, FGFR-2c, and FGFR-4—The cDNA encoding the extracellular domain of rat FGFR-1c, mouse FGFR-2c, or rat FGFR-4 with a DNA fragment encoding an E-tag (GAGFYVPYDPLEF) and a hexamer His tag (HHHHHH) at the 5’ terminus was constructed in a transfer vector DNA, *pBacPAK9* (CLONTECH). Recombinant baculovirus containing the cDNA was obtained by cotransfection of Sf9 cells with the recombinant *pBacPAK9* and a *BacPAK6* DNA (*Bac*36 digest) (CLONTECH). High Five cells (approximately 2 × 10^6 cells/ml) were infected with the recombinant baculovirus and incubated at 27 °C for 4 h in serum-free medium EX-CELL 400 (*JRH Biosciences*). The culture medium was dialyzed against phosphate-buffered saline (PBS) and applied to a column of Ni^{2+}-nitrilotriacetic acid-agarose (QIAGEN) in PBS containing 20 mM imidazole and 0.5 mM NaCl. After washing the column with PBS containing 20 mM imidazole and 0.5 mM NaCl, the recombinant extracellular domain was eluted from the column with PBS containing 250 mM imidazole and 0.5 mM NaCl and dialyzed against PBS.

Binding of FGF-16 to the Recombinant Extracellular Domains of FGFR-1c, FGFR-2c, and FGFR-4—FGF-16 as a control was fixed on the sensor tip CMS (Amersham Pharmacia Biotech). Binding of the recombinant extracellular domain of FGFR-1c, FGFR-2c, or FGFR-4 to the FGF on the tip was analyzed using the BIACORE 2000 system (Amersham Pharmacia Biotech). The equilibrium dissociation constant was determined by the BIA evaluation software (Amersham Pharmacia Biotech).

Cold Exposure—Wistar rats (8 weeks old) were kept at 4 °C for 1 h, 4 h, 24 h, and 1 week in individual cages with a 12-h light/12-h dark schedule as described (10).

RESULTS

Expression of FGF-16 mRNA in Rat Embryonic Brown Adipose Tissue—We examined the expression of FGF-16 mRNA and *UCP*-1 mRNA in brown adipose tissue at different developmental stages (embryonic days 19.5, 20.5, and 21.5 and postnatal day 1) by Northern blotting analysis. We also examined the expression of FGF-16 mRNA in rat brown adipose tissue at earlier developmental stages (embryonic days 16.5, 17.5, 18.5, and 19.5). Since it was difficult to prepare sufficient amounts of RNA from brown adipose tissue at these stages for Northern blotting analysis, we examined the expression of FGF-16 mRNA by *in situ* hybridization. Sagittal sections of rat embryos were analyzed by *in situ* hybridization with an ^3^S-labeled FGF-16 antisense cDNA probe (Fig. 2). FGF-16 mRNA was weakly detected in the interscapular area at embryonic day 16.5 (data not shown). However, FGF-16 mRNA was significantly detected in brown adipose tissue even at embryonic days 17.5 and 18.5 as well as embryonic day 19.5 (Fig. 2). The cellular localization of FGF-16 mRNA in brown adipose tissue was also examined. FGF-16 mRNA was detected in most cells in brown adipose tissue (data not shown).

Mitogenic Activity of Recombinant FGF-16 for Primary Rat Brown Adipocytes—Primary brown adipocytes were prepared from rat embryonic brown adipose tissue. Although the primary adipocytes were potentially heterogeneous, most of the cells differentiated into mature adipocytes (data not shown). We examined the mitogenic activity of recombinant rat FGF-16 for the primary adipocytes by determining [^3^H]thymidine incorporation. FGF-16 showed significant mitogenic activity for primary brown adipocytes (Fig. 3).

Expression of FGF Receptors in Rat Embryonic Brown Adipose Tissue—FGF's bind and activate FGF receptors. The cloning of FGF receptors has identified four distinct genes, FGFR-1 to FGFR-4 (19). The expression of FGF receptor mRNAs in rat embryonic brown adipose tissue at embryonic day 19.5 was examined by *in situ* hybridization. FGFR-1, FGFR-2, and FGFR-4 mRNAs, but not FGFR-3 mRNA, were found to be expressed in the brown adipose tissue (Fig. 4).

FGFR-1 to FGFR-3 have two alternatively spliced immunoglobulin-like domains, IIIb and IIIc. These spliced variants have different ligand specificities (19). Unlike FGFR-1 to FGFR-3, FGFR-4 is not alternatively spliced in this region. To identify the spliced variants of FGFR-1 and FGFR-2 expressed in the brown adipose tissue, we amplified FGF-1 and FGF-2 cDNAs encoding the immunoglobulin-like domain with the surrounding regions from the brown adipose tissue cDNA by polymerase chain reaction and examined the spliced variants of these receptors by determination of the FGF-1 cDNA sequence or the enzymatic digestion of the FGF-2 cDNA with *Hae* III. FGFR-1c and FGFR-2c, but not FGFR-1b and FGFR-2b, were found to be expressed in the brown adipose tissue (data not shown).

Receptor Specificity of FGF-16 in Embryonic Brown Adipocytes—To examine which FGF receptor (FGFR-1, FGFR-2, or FGFR-4) could bind FGF-16, recombinant extracellular do-

![Fig. 1. Expression of FGF-16 mRNA and UCP-1 mRNA in rat brown adipose tissue at different developmental stages (embryonic day 19.5 to postnatal day 1)]. 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 ^3^S-labeled rat FGF-16 or UCP-1 cDNA probe. RNA was also visualized with ethidium bromide. The positions of 28S and 18S rRNAs are indicated as 28S and 18S. Lanes E19.5, E20.5, E21.5, and P1 indicate RNA from brown adipose tissue at embryonic days 19.5, 20.5, and 21.5 and postnatal day 1, respectively.
mains of these receptors produced in insect cells were prepared. Since FGF-2 was shown to efficiently bind to FGFR-1c, FGFR-2c, and FGFR-4 (19), we also examined the binding of FGF-2 to recombinant extracellular domains of these FGF receptors. All three extracellular domains were found to bind FGF-2 with high affinity (Table I). The binding of FGF-16 to these receptors was also examined. In contrast to FGF-2, FGF-16 was found to bind to only the extracellular domain of FGFR-4 with high affinity and not to those of FGFR-1c and FGFR-2c (Table I).

Expression of FGF-16 in Brown Adipose Tissue

Wistar rats (8 weeks old) were kept at 4 °C (cold exposure) for 1 week. The expression of FGF-16 mRNA and UCP-1 mRNA in the brown adipose tissue after 1 week of cold exposure was examined by Northern blotting analysis. The expression of UCP-1 mRNA in brown adipose tissue was greatly increased by cold exposure (Fig. 1). In contrast, the expression of FGF-16 mRNA was not increased but was rather decreased (Fig. 5). We also examined the expression of FGF-16 mRNA in brown adipose tissue after 1-, 4-, and 24-h cold exposure. The expression of FGF-16 mRNA was also not increased (data not shown).

DISCUSSION

FGF-16 was originally identified from rat heart by homology-based polymerase chain reaction (9). Although FGF-16 lacks a typical signal sequence, it was efficiently secreted, indicating that FGF-16 is a secretory protein. FGF-16 mRNA was predominantly expressed in heart among adult tissues examined (9). The expression profile was quite distinct from those of other...
FGF mRNAs. In rat embryos at embryonic day 19.5, FGF-16 mRNA was predominantly expressed in brown adipose tissue, indicating that FGF-16 plays a crucial role in embryonic brown adipose tissue (9).

Brown adipose tissue is a specialized tissue of mammals responsible for facultative thermogenesis (1). Proliferation and differentiation of brown adipose tissue occurs during the embryonic period (1, 12). UCP-1 is a marker-specific for brown adipose tissue differentiation. The expression of UCP-1 mRNA in brown adipose tissue was greatly induced from embryonic day 20 (1). Our present results of UCP-1 mRNA expression are consistent with those reported (1). These results indicate that differentiation of brown adipose tissue is greatly stimulated from embryonic day 17. Mitogenic activity in the brown adipose tissue anlage was maximal during embryonic days 17–19, and continued thereafter at lower levels into the neonatal period (11). Therefore, in the present study, we also examined the expression of FGF-16 mRNA in brown adipose tissue during these periods by in situ hybridization. FGF-16 mRNA was significantly expressed in most of the cells in brown adipose tissue at embryonic days 17.5, 18.5, and 19.5, and the expression gradually decreased along with brown adipose tissue differentiation as described above. The expression profile of FGF-16 mRNA is closely related with mitogenic activity in brown adipose tissue reported (11).

FGFs are local signaling molecules that act on proximal cells (3). Therefore, FGF-16 in brown adipose tissue should act on proximal cells within the tissue. As FGF-16 mRNA was significantly expressed in brown adipose tissue at developmental stages when mitogenic activity in the brown adipose tissue anlage was maximal, we expected that FGF-16 was a mitogen for brown adipocytes. In the present paper, we examined the mitogenic activity of recombinant FGF-16 for primary rat brown adipocytes. FGF-16 showed significant mitogenic activity for primary rat brown adipocytes.

FGFs bind and activate FGF receptors. Four FGF receptor genes, FGFR-1 to FGFR-4, have been identified. Three of them have alternative spliced variants. Seven FGF receptors, FGFR-1b, FGFR-1c, FGFR-2b, FGFR-2c, FGFR-3b, FGFR-3c, and FGFR-4, with different ligand specificities have been identified (19). Among them, only three FGF receptors, FGFR-1c, FGFR-2c, and FGFR-4, were expressed in rat embryonic brown adipose tissue. However, only FGFR-4 could bind FGF-16. These results indicate that FGF-16 exerts the mitogenic activity by binding and activating FGFR-4 in the brown adipose tissue.

Cold exposure produces adaptive hyperplasia and growth of brown adipose tissue with an increase of several proteins specific for brown adipose tissue, such as UCP-1. The cellularity of brown adipose tissue (total tissue cell number) also greatly increases during cold acclimation. Radioautographic experiments using [3H]thymidine revealed that the mitogenic rate was maximal in brown adipose tissue during the first week of cold acclimation, declining thereafter (11). Therefore, we examined the expression of UCP-1 mRNA and FGF-16 mRNA in adult rat brown adipose tissue during cold exposure. The expression of UCP-1 mRNA in brown adipose tissue was greatly increased by cold exposure. In contrast, the expression of FGF-16 mRNA was not increased, but rather decreased, indicating that FGF-16 is not involved in adaptive hyperplasia and growth of brown adipose tissue during cold exposure.

Insulin-like growth factor-I was expressed in and induced the differentiation of embryonic brown adipose tissue, indicating that insulin-like growth factor-I is a growth factor involved in differentiation of embryonic adipose tissue (16). The proliferation and differentiation of brown adipose tissue during cold exposure are controlled by the sympathetic nervous system (14). However, the factors involved in the proliferation of embryonic brown adipose tissue have remained to be elucidated. The expression profile of FGF-16 mRNA and the activity of recombinant FGF-16 reported here indicate that FGF-16 is a unique growth factor involved in proliferation of embryonic brown adipose tissue.

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