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Molecules and Cells

Expression and Characterization of Fibroblast Growth Factor 8 from Mexican Axolotl, *Ambystoma mexicanum*

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Fibroblast growth factor (FGF) has been known to regulate the proliferation and differentiation of a variety of cell types via interaction with a specific FGF receptor on the cell surface. In the present study, *Fgf8* cDNA of Mexican axolotl, *Ambystoma mexicanum*, was expressed in *Escherichia coli* as an MBP-FGF8 fusion protein. The cell proliferation activity of the recombinant FGF8 (rFGF8) was measured by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazoliumbromide (MTT) assay. The addition of rFGF8 to the culture medium enhanced proliferation of BALB/c 3T3 and BHK21 cells about 1.4–1.5 fold. To analyze the binding activity of rFGF8 to the cell surface, cell surface enzyme linked immunosorbent assay was developed. Comparison of the structure of basic FGF with the computer-simulated structure of FGF8 suggested that Tyr-58, Glu-132, Tyr-139, and Leu-179 might be the potential receptor binding sites. Amino acid substitution muteins of FGF8 were constructed by PCR-derived directed mutagenesis and the muteins were overexpressed in *E. coli*. The rFGF8 muteins were purified and their binding activities were analyzed. Substitution of Tyr-58 or Glu-132 or Leu-179 of the FGF8 with alanine reduced the binding affinity, while substitution of Tyr-139 with alanine did not alter the binding affinity. These results imply that Tyr-58, Glu-132, and Leu-179 of FGF8 might be involved in its binding to the cell surface.

Keywords: Axolotl; FGF8; Mutein; Receptor.

Introduction

Fibroblast growth factors (FGFs) are known to regulate the proliferation, differentiation, and migration of a variety of cell types and to promote wound repair. FGFs also act as a potent stimulator of angiogenesis and might be associated with numerous diseases, such as tumors, rheumatoid arthritis, and diabetic retinopathy due to unregulated angiogenesis (Folkman and Klagsbrun, 1987; Folkman and Shing, 1992). In many vertebral developmental processes, FGFs act as signaling molecules and FGF8, especially, is deeply involved in the early stage of organ development, such as in the brain and limb (Szebenyi and Fallon, 1999).

In amniotes and an anuran amphibian, *Xenopus laevis*, Fgf-8 cDNA's have been cloned and their expression domain has been localized to the apical ectodermal ridge (AER) of the developing limb bud, indicating that FGF8 is an AER-specific signaling factor in limb development (Christen and Slack, 1997; Crossley and Martin, 1995; Vogel *et al*., 1996). However, in a urodele amphibian species, *Ambystoma mexicanum* (Mexican axolotl), the Fgf-8 expression domain has been mainly localized to the mesenchymal tissue of the developing limb bud and the regenerating limb. The distinctive expression pattern of Fgf-8 in axolotl raises the possibility that it plays a critical role in the process of regeneration considering the superb regenerative capacity in this species. The cloned axolotl FGF8 shows a high sequence similarity to FGF8s in other species with 76–88% identities in the deduced amino acid sequence. Furthermore, axolotl FGF8 contains a typical consensus amino acid sequence in the conserved core region of all members of the FGF family from *Xenopus* to human (unpublished results).

The FGF family consists of at least 19 related genes (FGF1–FGF19). All FGFs share a conserved central region of about 120 amino acids. A number of three-dimensional structures of acidic FGF (aFGF, FGF1) and basic FGF (bFGF, FGF2), including two acidic

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Abbreviations: AER, apical ectodermal ridge; BHK21, baby hamster kidney 21; CELISA, cell surface enzyme linked immunosorbent assay; FGF, fibroblast growth factor; HSPGs, heparan sulfate proteoglycans.

forms and three basic forms (Eriksson *et al.*, 1991; Zhang *et al.*, 1991; Zhu *et al.*, 1991; 1993), are available. Zhu *et al.* (1991) reported that the conserved regions on aFGF and bFGF consist of 12 antiparallel β strands that form a structure with three-fold internal symmetry called a β-trefoil. Nine of these β strands contain highly conserved hydrophobic residues.

Members of this family are secreted and are thought to affect target cells by binding to and activating specific FGF receptor (FGFR) tyrosine kinases (Johnson and Williams, 1993). On the cell surface, FGF also binds to heparan sulfate proteoglycans (HSPGs) through the sulfated polysaccharide moiety (Burgess and Maciag, 1989; Moscatelli, 1987). HSPG or heparin binding to FGF has been shown to be important for optimal biological activity (Harper and Lobb, 1988) and to enhance the binding affinity of FGF to FGFR (Kiefer *et al.*, 1991; Pantoliano *et al.*, 1994; Roghani *et al.*, 1994). The FGF-receptor interactions result in a mitogenic and/or differentiation signal, depending on the cell types involved.

Four FGF receptors (FGFR1–FGFR4) bind to FGFs with various affinity. For example, 'b' and 'c' isoforms of FGFR1, produced by alternative splicing, and FGFR2 bind to aFGF and bFGF equally, whereas only the 'c' splice form of FGFR4 preferentially binds to aFGF (Pertovaara *et al.*, 1993). bFGF, FGF4, FGF8, and FGF9 show high affinity to FGFR2c, FGFR3c, and FGFR4 (MacArthur *et al.*, 1995; Ornitz *et al.*, 1996), while FGF7 and FGF10 specifically bind to FGFR2b (Christen and Slack, 1997; Xu *et al.*, 1998). During murine limb patterning, FGF8b and FGF8c activate FGFR3c and FGFR4c. FGF8b also activates FGFR2c. The 'c' forms of FGFR2, FGFR3, and FGFR4 are expressed in mesenchymal tissue (MacArthur *et al.*, 1995).

There are two important regions in human bFGF (hbFGF) that bind to FGFR1 (Baird *et al.*, 1988). These regions are located at amino acid residues 24–68 and residues 106–115. The region 24–68 has high affinity to heparin, so the region 106–115 on bFGF is considered a putative FGFR binding site. Springer *et al.* (1994) identified high- and low-affinity receptor binding surfaces on bFGF on the basis of site-directed mutagenesis. They suggested that amino acid residues Tyr-103, Leu-140, and Tyr-24 on bFGF comprise the primary high-affinity binding surface, whereas amino acid residues Lys-110, Tyr-111, and Trp-114 comprise the secondary low-affinity binding surface. In addition, Zhu *et al.* (1995) identified amino acid Glu-96 on bFGF as a crucial residue for binding to FGFR1 by employing site-directed mutagenesis.

Here, we describe the cell proliferation–stimulating effect of recombinant axolotl FGF8, expressed in *E. coli*, on mouse BALB/c 3T3 cells and baby hamster kidney 21 (BHK21) cells using 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. We also report site-directed mutagenesis studies on the putative receptor binding sites on FGF8. Each of the amino acids comprising the potential receptor binding sites was mutated individually to alanine. Cell surface enzyme linked immunosorbent assay (CELISA) was used to compare the muteins with wild type for their cell surface binding affinity on 3T3 cells.

Materials and Methods

Cell culture BALB/C 3T3 and BHK21 cells were cultured at 37°C in Dulbecco's modified Eagle's medium (DMEM, GIBCO BRL) supplemented with 10% (v/v) fetal bovine serum (FBS, GIBCO BRL) under 5% (v/v) CO_2.

Construction of recombinant plasmids The *Fgf8* cDNA cloned in pGEX-4T-3 (Pharmacia) was digested with *Bam*HI and *Sal*I (NEB). After gel electrophoresis, the *Bam*HI/*Sal*I digested DNA fragment was purified from the gel slice by using GenecleanII Kit (Bio101). The pMAL-c2 vector (NEB) designed for high-level gene expression in *E. coli* using the *tac* promoter was digested with *Bam*HI and *Sal*I. The purified *Fgf8* cDNA was ligated into the pMAL-c2 and the recombinant plasmid carrying *Fgf8* was named pMAL-FGF8wt.

Purification of rFGF8 An overnight culture of the cells carrying the recombinant pMAL-c2 was inoculated into fresh Luria-Bertani (LB) broth in the presence of ampicillin (50 μg/ml). When the density of the culture reached 0.7–0.8 at OD_{600}, the *tac* promoter was induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.1 mM. After 3-h incubation at 37°C, the cells were harvested by centrifugation at 4,000 × *g* for 15 min at 4°C. The cell pellet was resuspended in 20 ml of STE (0.2 M sodium chloride, 0.1 M Tris-HCl, pH 8.0, 2 mM EDTA) per 400 ml of the original culture volume. The cell suspension was lysed with sonication three times and centrifuged at 10,000 × *g* for 20 min at 4°C. The supernatant collected was loaded onto a column containing amylose resin. After washing the column with phosphate-buffered saline (PBS), proteins were eluted with elution buffer (10 mM maltose in PBS). The purified MBP fusion protein was cleaved with Factor Xa protease and the cleaved-MBP was removed by anion-exchange chromatography using diethylamino ethyl (DEAE) Sepharose (Pharmacia).

MTT assay Cells were seeded at a density of 3×10^3–5×10^3 cells per well in 96-well plates (NUNC) and were cultured in DMEM supplemented with 10% FBS. After 24 h, the culture media were replaced with serum-free media (SFM) and rFGF8 was added. The cells were incubated for an additional 24 h, and then 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) stock solution (5 mg/ml in PBS) was added to the final concentration of 0.8 mg/ml. After 4 h incubation at 37°C, dimethyl sulfoxide was added to the wells and mixed thoroughly to dissolve dark-blue crystals. The plates were read on an ELISA reader SPECTRA MAX 250 (Molecular Devices), using a test wavelength of 540 nm and a reference wavelength of 650 nm.

Western blot Proteins were separated under reducing conditions on a 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The resolved proteins were electrotransferred onto a nitrocellulose membrane (Schleicher & Schuell) as described by Kang *et al*. (1999) at 15 V for 2–3 h in a transfer buffer (125 mM Tris-HCl, pH 7.8, 192 mM glycine, 20% methanol, 0.1% SDS). The membrane was blocked by 6% skimmed milk in Tris-buffered saline/Tween20 (TTBS, 100 mM Tris-HCl, 0.95% NaCl, 0.5% Tween20, pH 7.5) for 1 h. The nitrocellulose membrane was incubated with antisera diluted to 1:10,000 in blocking buffer (6% skimmed milk in TTBS) for 1 h. After washing the membrane with washing buffer (TTBS), goat anti-rabbit IgG antibody conjugated with alkaline phosphatase (Pierce) was added at a dilution of 1:5,000 for 40 min. After three washes with the washing buffer, immunodetection was accomplished by adding nitro-blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate.

Site-directed mutagenesis To construct amino acid substitution muteins of *Fgf8*, an overlapping method using a polymerase chain reaction (PCR) was applied (Kim and Yang, 1996). The sequence and the location of the primers used for mutagenesis are listed in Table 1. The PCR was performed in two separate tubes. One contained M1 (mismatch primer for mutation) and P2 primer, the other contained M2 (mismatch primer for mutation) and P1 primers. The PCR reaction mixture (100 μl) consisted of 20 pmol of each primer, each of 0.2 mM dNTPs, 100 ng of template, 10 μl of 10 × PCR buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl, 15 mM $MgCl_2$, 0.01% gelatin), and 2.5 units of ExTaq DNA polymerase (Takara). After initial denaturation at 95°C for 5 min, 30 cycles of denaturation at 95°C for 1 min, annealing at 50°C for 1 min, and polymerization at 72°C for 1 min were performed in a GeneAmp PCR System 2,400 TM (Perkin–Elmer), followed by final polymerization at 72°C for 5 min. The PCR products were then separated on a 1.3% agarose gel electrophoresis and eluted for purification. In a new tube, the reaction mixture, containing 100 ng of the PCR products, 20 mM Tris-HCl, pH 8.7, 40 mM KCl, 1 mM $MgCl_2$, each of 0.2 mM dNTPs, and 2.5 units of ExTaq was mixed for the overlap extension reaction. The PCR was processed under the same conditions as described earlier. Both the mutated PCR products and pMAL-c2 DNA were digested with *Bam*HI and *Sal*I. The *Bam*HI/*Sal*I restriction fragment of the PCR product bearing the desired mutation was ligated into the *Bam*HI/*Sal*I digested pMAL-c2 fragment.

Cell surface enzyme linked immunosorbant assay To BALB/C3T3 cells, grown in a 96-well plate at a density of 1×10^4, rFGF8 was added and the plate was incubated at 4°C for 4 h. The media were replaced with PBS containing 4% paraformaldehyde. After 30 min, fixed cells were washed with Tris-buffered saline (100 mM Tris-HCl, 0.95% NaCl, pH 7.5) and blocked with blocking buffer (6% skimmed milk in TTBS) for 1 h. Antisera diluted into 1:1,000 with blocking buffer was added and the plate was incubated for 1 h. After three washes with the washing buffer (TTBS), the plate was incubated with anti-rabbit IgG conjugated with horseradish peroxidase for 40 min. The plate was washed three times and developed with 150 μl of 0.02% H_2O_2 and 0.3% *o*-phenylenediamine in 100 mM citrate–phosphate buffer, pH 5.5. The OD at 405 nm was measured within 1 h using the ELISA reader.

Results

Expression of the rFGF8 *Fgf8* cDNA of Mexican axolotl, *Ambystoma mexicanum*, was cloned from an axolotl embryo cDNA library (Han, 1997). The 1.27-kb cDNA contains an open reading frame encoding putative polypeptide with 212 amino acids. In order to investigate the biological properties of FGF8, a large amount of rFGF8 was expressed in *E. coli* using the pMAL-c2 vector. Since the FGF8 cDNA was inserted at the 3′ end of the *malE* coding region, it should be expressed as an MBP-FGF8 fusion protein.

The *tac* promoter on the pMAL-FGF8wt vector was induced by the addition of IPTG. Proteins were separated on a 10% SDS-PAGE and visualized by staining with Coomassie blue. Lane 3 of Fig. 1 shows a new band with a molecular mass of about 65 kDa after induction with IPTG. This new band is presumed to be the MBP-FGF8 fusion protein since its molecular mass was estimated to be approximately 65 kDa using the PROSIS program (Hitachi Software Engineering).

Table 1. Sequence and position of primers used for PCR derived site-directed mutagenesis. Position 0 means the first nucleotide of pMAL-FGF8wt.

Primer	Sequence (5′ → 3′)	Size	Position (5′ → 3′)
mf8ss	CGCAGATGTCCGCTTTCTGGT	21	2531 → 2552
mf8so	GGATCCCCGAATTCAAAGGTAAC	23	2701 → 2724
mf8as	CAAGCTTGCCTGCAGGTCGAC	21	3296 → 3317
mf8aa	GCCATTCAGGCTGCGCAACTG	21	3449 → 3470
58s	CCTACCAGCTGGCCAGCCGCACCA	24	2807 → 2831
58as	TGGTGCGGCTGGCCAGCTGGTAGG	24	2807 → 2831
132s	CGTCTTCTCCGCCATAGTGCTCGA	24	3030 → 3054
132as	TCGAGCACTATGGCGGAGAAGACG	24	3030 → 3054
139s	TCGAGAACAACGCCACGGCACTAC	24	3050 → 3074
139as	GTAGTGCCGTGGCGTTGTTCTCGA	24	3050 → 3074
179s	TCATGAAGCGAGCCCCGAAAGGCCA	25	3170 → 3195
179as	TGGCCTTTCGGGGCTCGCTTCATGA	25	3170 → 3195

To determine the optimal incubation time for the maximal production of MBP-FGF8, the *E. coli* cells transformed with pMAL–FGF8wt were induced by the addition of IPTG and harvested after 1 h. The expression level of MBP-FGF8 reached a maximum at 3 h incubation after IPTG induction (Fig. 1, lanes 2–8).

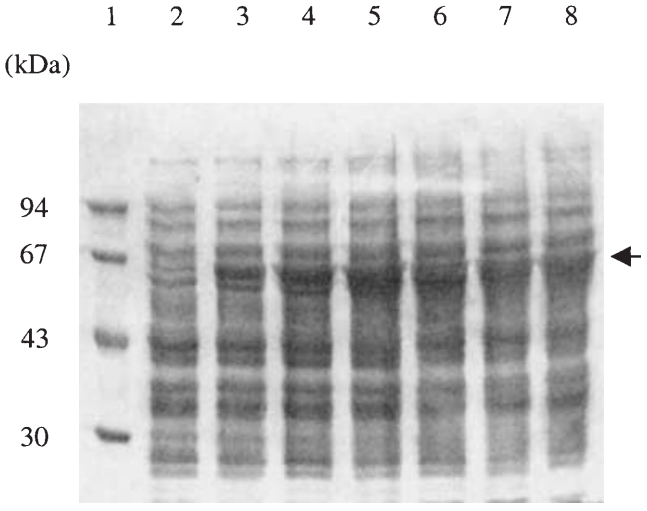

Fig. 1. SDS-PAGE of MBP-FGF8 expressed in *E. coli*. Lane 1, low-molecular-weight marker; lanes 2, 3, 4, 5, 6, 7, and 8, total cell extract harvested at 0–6 h in 1-h intervals, after induction with IPTG. The arrow indicates the induced fusion protein.

Purification of rFGF8 *E. coli* cells carrying the pMAL-FGF8wt vector were incubated for 3 h after addition of IPTG. The cells were harvested by centrifugation, resuspended in STE (0.2 M sodium chloride, 0.1 M Tris-HCl, pH 8.0, 2 mM EDTA), and the cell suspension was sonicated for cell lysis. After centrifugation of the cell lysate, the pellet and supernatant were subjected to gel electrophoresis. Although it was suggested that the MBP fusion might increase the solubility of the heterologous proteins expressed in *E. coli*, the MBP-FGF8 was expressed primarily in insoluble form (Fig. 2A, lanes 4 and 5).

Because the soluble form was expected to have its native conformation and to retain its biological properties, the supernatant was used for the purification of MBP-FGF8. Since MBP has a higher affinity to maltose compared to amylose, MBP fusion protein bound to amylose resin could be eluted from the column with maltose solution (Fig. 2A, lane 6). About 1.5 mg of MBP-FGF8 could be purified from 1.0 l culture.

The pMAL-c2 vector contains a sequence coding for the recognition site of a specific protease Factor Xa, located just 5′ to the polylinker insertion sites. This allows MBP to be cleaved from the protein of interest after purification. After MBP-FGF8 was treated with Factor Xa, two additional major protein bands, cleaved-MBP and cleaved-rFGF8, were detected (Fig. 2B, lane 3). The MBP was removed by anion-

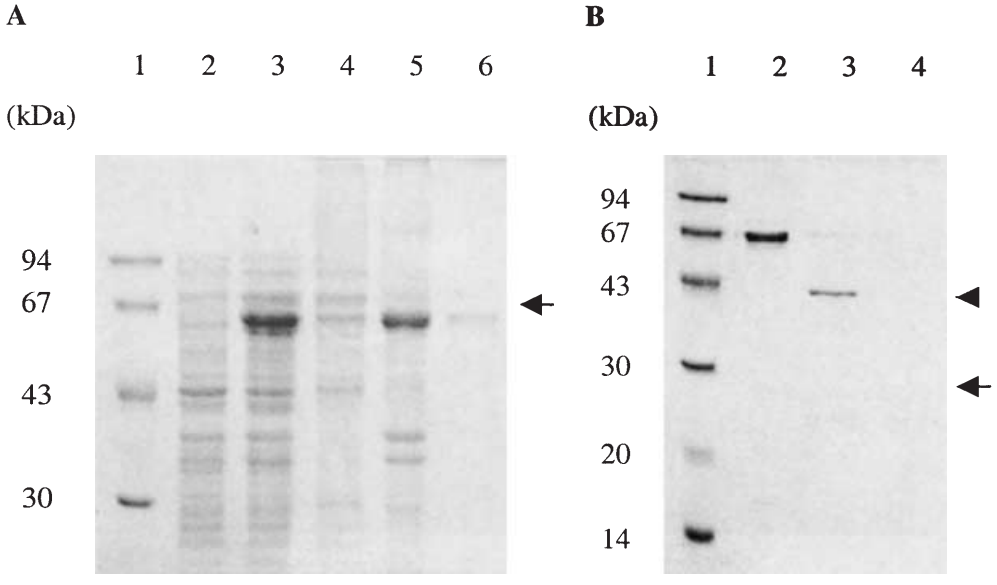

Fig. 2. SDS-PAGE of purified rFGF8. (**A**) The fusion protein, MBP-FGF8, was expressed in *E. coli* and purified by affinity chromatography using amylose resin. Lane 1, low-molecular-weight marker; lane 2, total extract of uninduced cells; lane 3, total extract of induced cells; lane 4, soluble fraction of induced cells; lane 5, insoluble proteins of induced cells; lane 6, purified MBP-FGF8. The arrow indicates the fusion protein. (**B**) The MBP-FGF8 was cleaved by factor Xa and purified by ion-exchange chromatography. Lane 1, low-molecular-weight marker; lane 2, MBP-FGF8 purified by affinity chromatography using amylose resin; lane 3, MBP-FGF8 treated with factor Xa; lane 4, rFGF8 purified by ion-exchange chromatography. The arrowhead indicates MBP. The arrow indicates rFGF8.

exchange chromatography using DEAE Sepharose (Fig. 2B, lane 4).

Cell proliferation activity Considering the unique expression pattern of axolotl Fgf-8 in the developing and regenerating limbs as well as the excellent regenerative capacity of axolotl, it is interesting to examine the molecular characteristics of axolotl FGF8; however, unfortunately, a cell culture system of axolotl to assay the molecular property of various growth factors has not been established yet. As an alternative, we decided to use the mouse BALB/C 3T3 cells and baby hamster kidney 21 (BHK 21) cells for the assay of axolotl FGF8.

The cell proliferation activity of rFGF8 was analyzed by MTT assay. Two mammalian fibroblast cell lines, BALB/C3T3 and BHK21 cells, were grown in DMEM supplemented with fetal bovine serum (FBS). Both cell lines did not grow detectable level in SFM; however, addition of rFGF8 to SFM enhanced proliferation of 3T3 and BHK21 cells about 1.5- and 1.4-fold, respectively (Fig. 3). FBS or commercially available recombinant human basic FGF (rhbFGF) also stimulated cell growth. While rhbFGF stimulated proliferation at a concentration of 2.5 ng/ml on BHK21 cells, a higher dose (1.5 μg/ml) of rFGF8 was required to get a similar proliferation effect.

Search for cell surface binding sites Tyr-24, Glu-96, Tyr-103, and Leu-140 of hbFGF were reported to comprise high-affinity receptor binding surfaces (Springer *et al*., 1994; Zhu *et al*., 1995). The amino acid sequences of hbFGF and axolotl FGF8 were compared to speculate on the putative ligand binding residues of FGF8. Tyr-58, Glu-132, Tyr-139, and Leu-179, respectively, of FGF8 matched with the corresponding amino acid residues of bFGF (Fig. 4). Analysis of the deduced amino acid sequence indicated that these four amino acid residues are conserved among the FGF8 family from zebrafish, *Xenopus*, chick, mouse, and human.

Figure 5 shows the three-dimensional structure of bFGF and the computer-aided predicted structure of FGF8. The four amino acids on bFGF compose a solvent-exposed cluster and Tyr-58, Glu-132, Tyr-139, and Leu-179 on FGF8 are located on the corresponding position. It was, therefore, assumed that these amino acid residues on FGF8 were most likely involved in receptor binding. Four amino acid substitution muteins of FGF8, Y58A, E132A, Y139A, and L179A, were constructed by PCR-derived mutagenesis. The muteins, overexpressed in *E. coli*, were purified by affinity chromatography using amylose resin and their cell surface binding activity was analyzed by CELISA. Substitution of Tyr-58, Glu-132, and Leu-179 into alanine reduced the binding affinity 0.5–0.6-fold while substitution of Tyr-139 into alanine did not affect the binding affinity (Fig. 6). These results imply that Tyr-58, Glu-132, and Leu-179 on FGF8 might play an important role in its cell surface binding activity.

Discussion

Fgf8 has been cloned in a number of vertebrate species, including human, mouse, chick, *Xenopus*, and fish

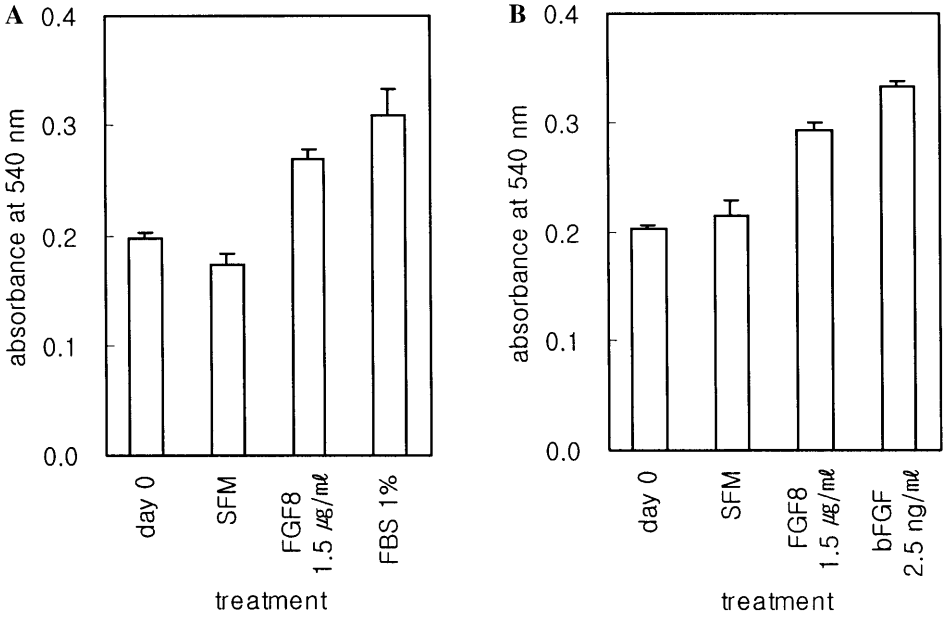

Fig. 3. Cell proliferation activity of the rFGF8. The numbers of 3T3 cells (**A**) and BHK21 cells (**B**) were determined by MTT assay after 1 d culture. SFM, serum-free media. FBS, fetal bovine serum. bFGF, recombinant human basic FGF. The vertical bars represent the mean of three cultures and the error bars represent the standard deviations.

(Christen and Slack, 1997; Crossley *et al.*, 1996; Fürthauer *et al.*, 1997; Gemel *et al.*, 1996; MacArther *et al.*, 1995). Han (1997) cloned an *Fgf8* cDNA of urodele species, Mexican axolotl, and the biological activity of rFGF8, expressed in *E. coli* as a GST-fusion protein, was tested in chick embryos before investigating its role in axolotl limb regeneration. rFGF8 induced generation of an additional limblike structure from the flank interlimb region, indicating that rFGF8 has biological activity.

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bFGF ----------------------------------------PALPEDGGSGA
FGF8 MNNESSSVVGYLLLHLFVLCMQAKVTVQSPPNFTQHVREQSLVTDQLS--

                24
bFGF FPPGHFKDPKRLYCKNGGFFLRIHPDGRVDGVREKSDPHIKLQLQAEERG
FGF8 ---RRLIRTYQLYSRTSGKQVQVLGNKKINAMAEDGDSHAKLIVETDTFG
                58
                                                96    103
bFGF -VVSIKGVCANRYLAMKEDGRLLA-SKCVTDECFFFERLESNNYNTYRSR
FGF8 SRVRIKGAETGYYICMNKKGKLIGKSNGRGKDCVFSEIVLENNYTALQNA
                                                132   139
                                           140
bFGF KYTSWYVALKRTGQYKLGSKTGPGQKAILF---LPMSAKS----------
FGF8 KYEGWYMAFTREGRPRKGSKTRQHQREVHFMKRLPKGHQTTEPHRRFEFV
                                           179

bFGF ----------------- 146
FGF8 NYPFNRRSKRTRHSVPR 212
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Fig. 4. Comparison of the deduced amino acid sequences of hbFGF with axolotl FGF8. Tyr-24, Glu-96, Tyr-103, and Leu-140 of the bFGF match with Tyr-58, Glu-132, Tyr-139, and Leu-179 of FGF8. Dashes (-) represent gaps inserted to align homologous residues using the Clustal W program. Identical amino acids are indicated by gray boxes.

We developed a cell proliferation assay system to evaluate the biological activity of FGF8 *in vitro*. MBP-FGF8 was expressed in *E. coli* and the protein lacking MBP was purified by anion-exchange chromatography. Bioassays done *in vitro* showed that rFGF8 has cell proliferation activity on BALB/C3T3 and BHK21 cells. A higher concentration of rFGF8 compared to rhbFGF was required to have a similar level of the cell proliferation effect. These results suggested that amphibian rFGF8 has a weak proliferation activity on mammalian fibroblast cells.

Springer *et al.* (1994) reported that bFGF has three surfaces: a primary receptor binding domain, a secondary FGFR binding domain, and a heparin binding domain. The highest variation in amino acid sequence between members of the FGF family is in the secondary binding domain. Substitution of amino acid residues on this domain resulted in reduced mitogenicity without showing any significant change in cell surface binding activity. This domain might determine the distinct receptor binding characteristics, tissue specificity, and the unique biological activity of FGFs. Since the *Fgf8* cDNA was cloned from the axolotl cDNA library, the sequence of the secondary receptor binding site on FGF8 might be different from that of mammalian FGF. This might be the reason why rFGF8 has weak proliferation activity in mammalian cells.

Several attempts have been made to identify the amino acids involved in the FGF-FGFR interactions. These attempts have mainly focused on aFGF and

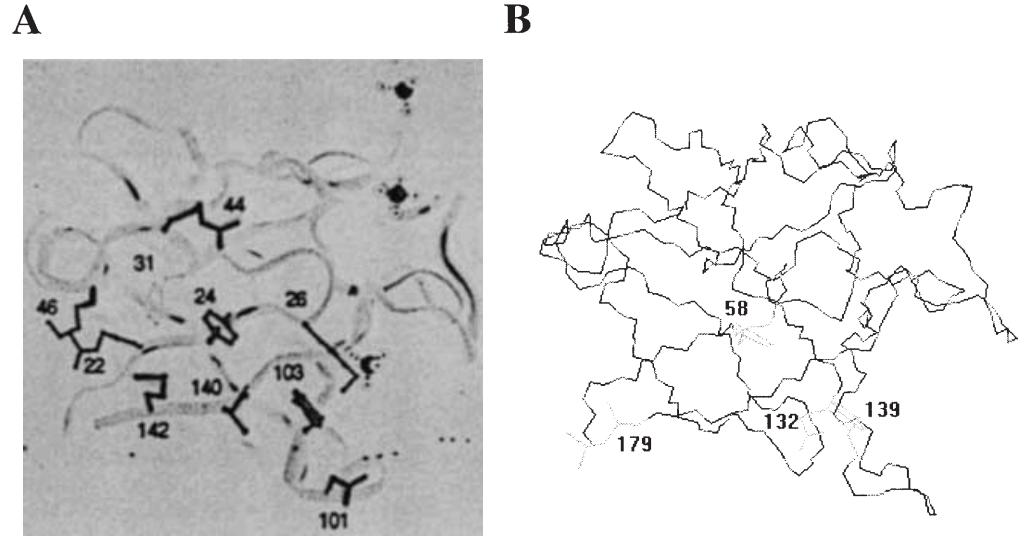

Fig. 5. Three-dimensional structures of hbFGF and axolotl FGF8. **A.** Ribbon diagram of the bFGF Cα backbone (Springer *et al.*, 1994). Amino acid side chains involved in receptor binding are displayed. **B.** The FGF8 Cα backbone predicted using SWISS-MODEL, an automated comparative protein modeling server. Amino acid side chains predicted to interact with its receptor are displayed.

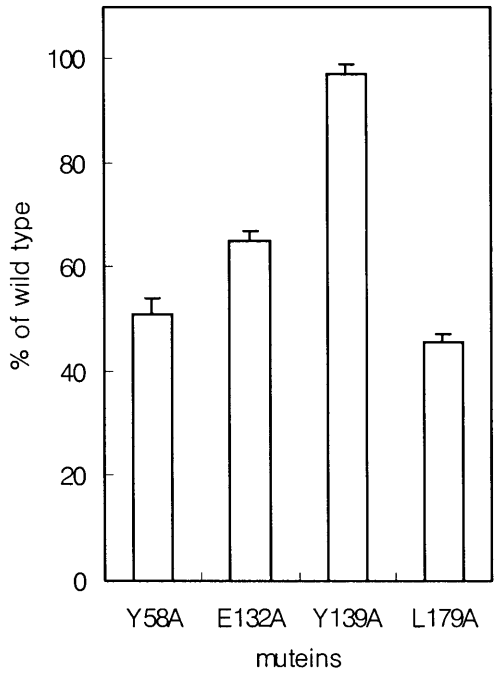

Fig. 6. Relative levels of cell surface binding activity of rFGF8 muteins. 3T3 cells were treated with MBP-FGF8 at the same concentration (5.0 μg/ml) and the level of cell surface bound MBP-FGF8 was analyzed by CELISA. The vertical bars represent the mean CELISA value (absorbance at 405 nm) of three cultures normalized to the mean CELISA value of rFGF8 wild type. The error bars represent the standard deviations.

bFGF, and there is no information about the receptor binding site on FGF8 at present. Generally, a peptide mapping is made followed by the study of the three-dimensional structure of a protein in order to define the ligand binding site on the protein. Reports on bFGF have accumulated and most residues that participate in receptor binding have been identified (Zhu *et al.*, 1998).

In this work, we employed site-directed mutagenesis based on the comparison of the deduced amino acid sequence of FGF8 with that of bFGF and the structure of FGF8 generated by an automated comparative protein modeling with the crystallographically determined structure of bFGF.

The primary receptor binding site of bFGF is discontinuous in the primary amino acid sequence and the affinity is dominated by hydrophobic interactions provided by amino acids Tyr-24, Tyr-103, Leu-140, and Met-142 (Springer *et al.*, 1994). Several polar amino acids are also involved in receptor binding, and the electrostatic interaction in Glu-132 of bFGF has been considered as an important component of receptor binding (Zhu *et al.*, 1995). Alignment of the deduced amino acid residues of bFGF and FGF8 indicated that Tyr-58, Glu-132, Tyr-139, and Leu-179 of FGF8 correspond with Tyr-24, Glu-96, Tyr-103, and Leu-140 of bFGF, respectively. The four amino acids on the proteins are located in similar positions in the predicted three-dimensional structures of the proteins. On the assumption that the four residues on FGF8 might be involved in receptor binding, site-directed mutagenesis was performed and muteins were tested for their binding affinity.

Substitution of Tyr-58, Glu-132, and Leu-179 of FGF8 into alanine resulted in a reduction of the cell surface binding affinity. Y139A mutein showed little decrease in the cell surface binding. These results suggest that the receptor binding sites on FGF8 might be similar to those on bFGF, but not identical. On the basis of the properties of the amino acids involved in the cell surface binding, both hydrophobic interaction and electrostatic interaction may be important for FGF8 receptor binding. Since the results of the MTT assay for the bioactivity of FGF8 were not high enough, the growth assay of the muteins was not performed. Further study is required to give an answer to the question of whether the loss of cell surface binding affinity of the muteins is due to a conformational change.

Considering the potential value of FGF8 as a signaling molecule in basic research and practical use, it would be very useful to establish an assay system to evaluate the efficacy of variants of FGF8. In that respect, establishing a cell culture system of axolotl is necessary to examine the molecular property of various muteins of FGF8 and other potential signaling molecules rigorously.

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