Overlapping Expression and Redundant Activation of Mesenchymal Fibroblast Growth Factor (FGF) Receptors by Alternatively Spliced FGF-8 Ligands*

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FGF-8 is a member of the family of fibroblast growth factors and is expressed during vertebrate embryo development. Eight potential FGF-8 isoforms are generated by alternative splicing in mice, several of which are expressed during embryogenesis in epithelial locations. The significance of the multiple isoforms is currently unknown. In this report, we investigate the expression patterns and the specificity of the FGF-8 isoforms for known fibroblast growth factor (FGF) receptors. RNAs for seven of the eight potential isoforms are present at multiple sites of embryonic Fgf8 expression. None of the FGF-8 isoforms exhibited activity when assayed with BaF3 cells expressing the “b” splice forms of FGF receptors 1–3, which are mostly expressed in epithelial tissues. Mesenchymally expressed “c” splice forms of FGF receptors 2 and 3 and FGF receptor 4 were activated by several FGF-8 isoforms. These findings are consistent with the hypothesis that the multiple FGF-8 isoforms are functionally redundant and function to signal in paracrine (epithelial to mesenchymal) contexts.

The mammalian fibroblast growth factor (FGF) family currently consists of structurally related polypeptides encoded by 10 genes (FGF-1–10) (reviewed in Ref. 1; see Refs. 2–4). Four distinct genes code for high affinity transmembrane receptor tyrosine kinases (FGFR1–4) that bind FGF ligands and display varying patterns of expression (reviewed in Ref. 5). Alternative mRNA splicing generates isoforms of receptors 1–3 that exhibit unique ligand binding characteristics (6–9). FGF receptor activation involves ligand binding and receptor dimerization, followed by transphosphorylation of the receptor and transduction of the signal into a biological response (5). FGF signal transduction has been implicated in development, wound healing, angiogenesis, and tumorigenesis (reviewed in Ref. 1). Germ line mutations of FGFs and FGFRs in mice (10–15) and humans (16–21) demonstrate the importance of FGF signaling in the process of development.

FGF-8 was first identified as an androgen-induced growth factor secreted by the mouse mammary tumor cell line SC-3 (2). Subsequently, Fgf8 was identified as a Wnt1-cooperating proto-oncogene in murine mammary tumorigenesis (22). Fgf8 expression has been detected during murine and chicken embryogenesis in regions of outgrowth and patterning, including the primitive epiblast, the apical ectodermal ridge of the limb bud, the primitive streak, the tail bud, the facial primordia, and the midbrain-hindbrain junction (22–29). The murine and human genes encoding FGF-8 have been localized to mouse chromosome 19 and human chromosome 10q24–26 (25, 26, 30, 31). The murine Fgf8 gene is unusual in the FGF family in that there are four exons (exons 1A–1D) equivalent to the usual exon 1 in other FGF genes (22, 25, 32). Alternative splicing of the four alternatively spliced exons results in potentially eight protein isoforms that differ at their amino termini and share a common carboxyl terminus encoded by exons 1D, 2, and 3 (22, 25, 32). Human FGF8 is similar to murine Fgf8 in structure; however, only four of the protein isoforms are encoded by FGF8 due to a blocked reading frame in the longer form of exon 1B (33).

The significance of the multiple FGF-8 protein isoforms is not clear. RNAs encoding several of these isoforms, or the protein isoforms, have been detected in the mouse embryo by ribonuclease protection and in situ hybridization assays (23, 32), demonstrating no significant difference in the expression patterns of the RNAs encoding the different FGF-8 isoforms. One exception is that RNA encoding FGF-8a was not seen in E10.5 tail bud (27). Also, multiple Fgf8 RNAs were detected by reverse transcription-polymerase chain reaction (RT-PCR) in the tail bud, limb bud, forebrain, and midbrain-hindbrain junction of E9.5 embryos, but RNA for FGF-8b was thought to be predominant (25). While there are discrepancies in the temporal and spatial expression of the various isoforms during development, there are some differences in their biological activity. FGF-8b potently transforms NIH 3T3 cells, while FGF-8a and FGF-8c do so weakly (34, 35). FGF-8b binds to and stimulates mitogenesis in cells containing FGFR3c > FGFR4 > FGFR2c, while FGF-8c interacts with FGFR3c > FGFR4 and does not interact with FGFR2c (32). Although FGF-8a had some activity in a limb bud assay (27), no activity for FGF-8a with any of the known FGFRs was demonstrated in vitro (32). FGF-8a, FGF-8b, and FGF-8c did not interact appreciably with FGFR1b, FGFR1c, FGFR2b, or FGFR3b, suggesting that epi-
thelially produced FGF-8 isoforms interact with mesenchymally located receptors (32).

We now show that at multiple sites of Fgf8 expression during murine embryogenesis, RNAs encoding seven of the eight potential FGF-8 protein isoforms are present, suggesting that there is little, if any, temporal or spatial regulation of FGF-8 isoforms during development. We further demonstrate that there are three classes of FGF-8 isoforms with respect to their interactions with FGRFs: FGF-8b, and FGF-8f, which interact with FGRFRc, FGRFr3, and FGRFr4; FGF-8e, FGF-8d, FGF-8e, and FGF-8g, which interact with FGRFRc and FGRFr4; and FGF-8a, which does not interact with any of the known FGRFs (32). These results suggest that the different FGF-8 isoforms are redundant.

EXPERIMENTAL PROCEDURES

RT-PCR Analysis of Fgf8 Expression during Embryogenesis—Swiss Webster mouse embryos were obtained from timed matings (B&K Universal, Edmonds, WA). Noon on the day the copulation plug was found was considered to be 0.5 days post-coitum (E0.5). Individual embryos were staged at the time of harvest according to Ref. 36. Microdissection of the embryo parts was performed, and total RNA was prepared from the embryonic tissues (Ultraspec RNA isolation system, Biotex Laboratories, Houston, TX).

First-strand cDNA was synthesized from 4 µg of total RNA in 33-µl reactions using random hexamer primers as described in the first-strand cDNA synthesis kit (Pharmacia Biotech Inc). The entire first-strand reaction was amplified by PCR in 100-µl reactions using random hexamer primers as described in the first-strand cDNA synthesis kit (Pharmacia Biotech Inc). The entire first-strand reaction was amplified by PCR in 100-µl reactions using 2.5 units of Taq DNA polymerase (Life Technologies, Inc.) and the following Fgf8 primers at a final concentration of 0.1 µM each: forward, 5′-TCGACCATCCGCTGGTGC-3′; and reverse, 5′-CGAGTCCCGGGT- GGATTCT-3′. Thermal cycling (PTC-100, M, Research, Inc.) parameters were as follows: 95°C for 2.5 min, 61°C for 50 s, and 72°C for 1 min for the initial cycle, followed by 34 cycles of 95°C for 50 s, 61°C for 50 s, and 72°C for 1 min, and then a 7-min extension at 72°C and a 4°C hold. PCR products were analyzed on 3% agarose (NuSieve) gels and 8% polyacrylamide gels in Tris borate/EDTA buffer.

The observed PCR fragments were gel-purified and cloned into Blue-script KS+ plasmids (Stratagene) by TA cloning (37). The inserts were sequenced by thermal cycle sequencing with 32P-end-labeled primers (T3, T7, and Fgf8-specific) and the fmol 32P kit (Promega). The resulting sequencing products were analyzed with MacVector version 5.0 software (Kodak Scientific Imaging Systems).

Preparation of Recombinant FGF-8 Isoforms—Full-length cDNAs for FGF-8d, FGF-8e, FGF-8f, and FGF-8g isoforms were generously provided by Philip H. Crossley and Gill R. Martin and correspond to variants 3, 5, 6, and 5, respectively (25). cDNAs encoding the mature FGF-8 isoforms (lacking the signal peptide and the stop codon) were obtained by PCR methods as described previously for preparation of isoforms FGF-8a, FGF-8b, and FGF-8c (32). The following forward primers were used: for FGF-8d and FGF-8g, 5′-AAAGGATCCTCAAGGGGCTGCCGGCC-3′; and for FGF-8e and FGF-8f, 5′-AAAGGATCCTCAAGGGGCTGCCGGCC-3′. The following reverse primer was used for all of the isoforms: 5′-AAAGGATCCTCAAGGGGCTGCCGGCC-3′. PCR was performed under the following conditions: 30 cycles at 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min, followed by a 10-min extension at the end of cycling at 75°C. Recombinant Pfu DNA polymerase (Stratagene) was used with 1× Pfu buffer, (2 mM MgCl2), 0.2 mM dNTPs, 1 µM concentrations of each primer, and 10 ng of target cDNA. The amplified products were electrophoresed, purified from agarose gel, and digested with BamHI and EcoRI, and ligated into the vector pQE6 (QIAGEN Inc.).

Each plasmid containing the appropriate FGF-8 coding region was used to transform XL-1 Blue bacteria (Stratagene), followed by sequencing of the plasmid DNA to confirm that there were no mutations. Mutation-free plasmids containing FGF-8 regions were then transfected into the M15 or SG13009 strain of Escherichia coli (QIAGEN Inc.). Mutation-free plasmids containing FGF-8 regions were then transfected into the M15 or SG13009 strain of Escherichia coli (QIAGEN Inc.).

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Analysis of FGF-8 Isoform Location during Mouse Development—Fgf8 encodes eight potential protein isoforms, seven of which have been identified (25) by alternative splicing of exons 1A–1D (Fig. 1). The presence of multiple FGF-8 isoforms encoded by a single gene raises the question of whether the isoforms might have unique temporal or spatial expression patterns or unique abilities to interact with FGRFs. Prior attempts to analyze the temporal and spatial expression patterns of the different FGF-8 isoforms have generated conflicting results (23, 25, 26, 32). Since in situ hybridization and immunohistochemical approaches are limited by the availability of unique reagents, we chose to analyze by RT-PCR dissected embryo tissues from several gestational stages for the presence of RNAs that encode FGF-8 isoforms. E7.5 mid-streak embryo; E8.5 forebrain and midbrain; E9.5 prosencephalon and tail bud; and E10.5 maxillary arch, nasal placode, isthmus, and fore limb buds all contained eight bands when analyzed on ethidium bromide-stained polyacrylamide gels (Fig. 2). The bands were further identified as cDNAs encoding FGF-8 isoforms by cloning and sequencing of the observed PCR bands (data not shown). The lower seven bands corresponded to the prior identified Fgf8a–g isoforms (25). The upper eighth band corresponded to a partially spliced RNA that has the intron between the longer form of exon 1B and exon 1C in the RNA that would otherwise encode FGF-8f (Fig. 1). This upper band would result in a translation reading frameshift that would not encode a FGF-8 isoform. This RT-PCR is not “quantitative,” but the results indicate that RNAs for seven of the eight possible FGF-8 proteins are produced when Fgf8 is expressed during mouse development.

Ligand-Receptor Interactions of the FGF-8 Isoforms—The RNA localization studies (Fig. 2) and prior work (23, 25, 26, 32) indicate that multiple FGF-8 isoforms are produced when Fgf8 is expressed. The apparent absence of different expression patterns raises the possibility that the different FGF-8 isoforms may bind to and activate different FGRFs. We have developed a mitogenic assay for FGF activity in BaF3 cells expressing a
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**FIG. 1. Structure of the murine Fgf8 gene.** Murine Fgf8 consists of at least six exons, which code for eight potential isoforms. Exons that code for regions common to all of the mature FGF-8 isoforms ( ), exons that code for the shared signal peptide ( ), exons that are alternatively spliced and code for regions that are part of some of the FGF-8 isoforms ( ), and noncoding exons ( ) are indicated. The exons coding for the potential FGF-8 isoforms are indicated below the representation of the genes. The FGF-8h isoform is only theoretical and has not been identified. The human FGF8 gene encodes only FGF-8a, FGF-8b, FGF-8e, and FGF-8f (33).

**FIG. 2. RNAs for seven of the eight possible murine FGF-8 isoforms are present at multiple sites of Fgf8 expression.** Shown is an ethidium bromide-stained 8% polyacrylamide gel of RT-PCR samples of RNAs prepared from dissected mouse embryos. The gestational age of the embryo is indicated above the gel (in days post-coitum, i.e. E9.5). The embryo parts are given above the gel: fl, fore limb bud; mb, nasal pits and maxillary arch; isth, isthmus (midbrain-hindbrain junction); fb, forebrain and midbrain; pro, prosencephalon; th, tail bud; m/a, nasal pits and maxillary arch; i, isthmus (midbrain-hindbrain junction); f, fore limb bud. The different isoforms are depicted to the right of the gel (a–g). X is a partially spliced form of FGF-8f. All bands were cloned and sequenced to confirm their identity.

The carboxyl-terminal end because this site was the same for all isoforms and would be unlikely to affect one isoform selectively. We have shown that for the FGF-8h isoform, the carboxyl-terminal tag and the absence of glycosylation in the recombinant isoform do not affect receptor specificity when compared with the native isoform (32). The recombinant FGF-8 isoforms were of the correct predicted size as assessed by SDS-polyacrylamide gel electrophoresis (Fig. 3). Additionally, the cDNA constructs were sequenced to confirm that there were no mutations. These recombinant FGF-8 isoforms were used in the BaF3 mitogenic assay. None of the FGF-8 isoforms was able to induce a mitogenic response in BaF3 cells expressing FGFR1b, FGFR1c, FGFR2b, or FGFR3b; however, these BaF3 cell lines all exhibited a good mitogenic response when incubated with FGF-1 (Fig. 4, A–C and E). BaF3 cell lines expressing FGFRC2c and FGFR3c responded to some of the FGF-8 isoforms and to FGF-1 (Fig. 4, D and F). FGFR2c-expressing cells responded well to FGF-8f and weakly to FGF-8d, but did not respond to FGF-8e and FGF-8g (Fig. 4D). FGF-8d, FGF-8f, and FGF-8g all activated FGFR3c-expressing BaF3 cells to a similar extent, whereas FGF-8e also showed activity, but was only slightly more than half that of the other three isoforms (Fig. 4F). All four FGF-8 isoforms as well as FGF-1 were able to induce a mitogenic response in BaF3 cells expressing FGFR4 (Fig. 4G).

FGF-1 induces mitogenesis in all BaF3 cell lines (Fig. 4) (8, 9, 32, 39). To compare the potency of the various FGF-8 isoforms with the different splice forms of the receptors, we normalized the FGF-8 data of Fig. 4 to that of FGF-1. Relative mitogenic activity was calculated at two points on each curve, and the two values were averaged and plotted in Fig. 5. Data for FGF-8a, FGF-8b, and FGF-8c are from earlier work (32). There are three classes of FGF-8 isoforms with respect to their abilities to activate FGFRs: Class 1, containing FGF-8c, FGF-8d, FGF-8e, and FGF-8g, which induce mitogenesis in cell lines expressing FGFR3c and FGFR4; Class 2, containing FGF-8b, and FGF-8f, which induce mitogenesis with FGFR2c, FGFR3c, and FGFR4; and Class 3, containing FGF-8a, which has no activity in this assay (Fig. 5).

**DISCUSSION**

The alternative splicing of Fgf8, resulting in multiple protein isoforms, is unique in the FGF family, and the role of the different isoforms is unclear. One possibility is that the different FGF-8 isoforms are expressed in different temporal and/or spatial contexts. A second possibility is that different FGF-8 isoforms bind to and activate different FGFRs. A third possibility is that the different FGF-8 isoforms are redundant and serve a unique role. If this latter possibility is true, any regulation of FGF-8 signaling would be at the level of receptor expression. Our results (Fig. 2) and those in the literature (25, 32) indicate that at multiple sites of Fgf8 expression, RNAs encoding all known FGF-8 isoforms are present. It is possible that subtle quantitative differences in the steady-state levels of RNA encoding FGF-8 isoforms exist (25, 27), but the significance of potential differences remains to be determined. Al-
FIG. 4. Recombinant FGF-8 isoforms induce mitogenesis in BaF3 cells expressing FGFR2c, FGFR3c, or FGFR4. BaF3 cells, stably transfected with and expressing FGFR1b (A), FGFR1c (B), FGFR2b (C), FGFR2c (D), FGFR3b (E), FGFR3c (F), or FGFR4 (G), were treated with
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though some regulation of alternative splicing may exist, the regulation is not absolute, and there is no discernible temporal or spatial regulation of FGF-8 isoform expression.

Given the apparent lack of temporal or spatial differences in FGF-8 isoform location, we examined the different FGF-8 isoforms for mitogenic activity in cells expressing defined FGFRs. FGF-8b and FGF-8f are similar in their activation of FGFRs, stimulating mitogenesis with FGFR2c, FGFR3c, and FGFR4 (Figs. 4 and 5). FGF-8c, FGF-8e, and FGF-8g activate FGFR3c and FGFR4, but not FGFR2c (Figs. 4 and 5). FGF-8d is similar to FGF-8b and FGF-8f, but it only weakly activates FGFR2c (Figs. 4 and 5). These results indicate that rather than activating distinct receptors, the FGF-8 isoforms are very similar in activity. None of the isoforms activate the "b" splice forms of FGFR1–3 (Figs. 4 and 5), demonstrating that one role of FGF8 in development is to signal in a paracrine fashion from epithelial (site of Fg8 expression) to mesenchymal (location of "c" splice forms of FGFR2 and FGFR3) regions (32).

From a structure-function view, it appears that the 11 amino acids encoded by the 5′-region of the longer form of exon 1D (Fig. 1) are responsible for the ability of FGF-8 isoforms to activate FGFR2c. The isoforms that contain these 11 amino acids (FGF-8b, FGF-8d, and FGF-8f) (Fig. 1) are the only isoforms that activate FGFR2c (Figs. 4 and 5). We hypothesize that if FGF-8x exists, it would also have some activity for FGFR2c.

These assays were performed at nanomolar concentrations of FGF-8 (Fig. 4). It is not clear what the physiologically relevant concentration of FGF-8 isoforms is during development. However, we (32) and others (34) have shown some activity of FGF-8 isoforms on FGFR1c when the concentration of FGF-8 is in the micromolar range. The importance of this weaker interaction for normal development remains to be determined.

FGF-8b has recently been shown to induce, initiate, and maintain the development of the chick limb (28) and has been shown to induce an ectopic midbrain when placed in the caudal forebrain of the chick embryo (29). As Fgf8 is expressed normally at these locations in the chick (28, 29) and mouse (23–25, 27), it is likely that one or more of the FGF-8 isoforms normally perform these functions during vertebrate development. Given that FGF-8b can perform these functions (28, 29) and that it is highly conserved evolutionarily (mouse and human, 100% identical; chicken, 82% identical) (28, 33), it seems likely that it is an important FGF-8 isoform. FGF-8f has very similar activity to FGF-8b in our mitogenic assays and is nearly as well conserved evolutionarily as FGF-8b (mouse and human, 97% identical) (33). The role of the other FGF-8 isoforms is less clear. FGF-8c, FGF-8d, FGF-8g, and FGF-8h are not encoded by human FGF8 (33). FGF-8a and FGF-8e are the least potent isoforms in our assays, but they may have roles in non-mitogenic contexts or with as yet undiscovered FGFRs. The possibility of heterodimerization of FGF-8 ligands and their effects on FGFR signaling remain to be determined.

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rFGF-1 (○), rFGF-8d (■), rFGF-8e (○), rFGF-8f (×), or rFGF-8g (●) in the presence of 1H]thymidine, and 1H incorporation into DNA was measured (counts/minute) and is plotted versus concentration of rFGF. The data points are the mean of two determinations, and the standard deviation is indicated by error bars.
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