INTRODUCTION

The mammalian Fibroblast Growth Factor (FGF) family consists of at least nine related genes that are classified as members on the basis of conserved coding sequence (Tanaka et al., 1992; Miyamoto et al., 1993; reviewed by Basilico and Moscatelli, 1992). Most FGFs are secreted and are thought to affect target cells by binding to and activating members of the high-affinity FGF receptor (FGFR) family, encoded by four mammalian genes (reviewed by Johnson and Williams, 1993). The result of these ligand-receptor interactions is a mitogenic and/or differentiation signal, depending on the cell type involved. While FGFs have many functions in vitro, one of their in vivo functions is to provide appropriate signals necessary for correct vertebrate development. In Xenopus, FGF signaling is involved in the induction of posterior mesoderm and can be inhibited by the overexpression of a dominant negative FGFR1 (Amaya et al., 1991). Mice homozygous for a null Fgrf1 allele die in utero and show disordered mesoderm patterning, implicating FGF signaling in mammalian axis determination (Yamaguchi et al., 1994; Deng et al., 1994). FGF signals from the apical ectodermal ridge (AER) of the developing vertebrate limb are necessary for normal outgrowth and patterning of the limb (Niswander et al., 1993; Fallon et al., 1994; Niswander et al., 1994; Cohn et al., 1995).

Fgf8 was originally identified as the gene encoding two secreted androgen-induced growth factors that were responsible for the androgen-dependent growth of the SC-3 mammary carcinoma cell line (Tanaka et al., 1992). One of these FGF-8 proteins can bind to and activate a mutant form of FGFR1 (Sato et al., 1993), and can transform NIH3T3 cells (Kouhara et al., 1994). Subsequently, Fgf8 was identified as a Wnt1-cooperating proto-oncogene in mammary tumorigenesis (MacArthur et al., 1995a). Fgf8 is expressed in several areas of the developing mouse that direct the outgrowth and patterning of the embryo (Heikinheimo et al., 1994b; Ohuchi et al., 1994; Crossley and Martin, 1995). These regions include the elongating body axis, midbrain/hindbrain junction, limb and face.

Fgf8 is expressed in developing limb and craniofacial structures, regions known to be important for growth and patterning of the mouse embryo. Although Fgf8 is alternatively spliced to generate at least 7 secreted isoforms that differ only at their mature amino terminus, the biological significance of these multiple isoforms is not known. In this report, we demonstrate that multiple FGF-8 isoforms are present at sites of Fgf8 expression during mouse development. To address the possibility that the FGF-8 isoforms might interact with different fibroblast growth factor receptors, we prepared recombinant FGF-8 protein isoforms. We examined the ability of these proteins to activate alternatively spliced forms of fibroblast growth factor receptors 1-3, and fibroblast growth factor receptor 4. Recombinant FGF-8b and FGF-8c activate the ‘c’ splice form of FGFR3, and FGFR4, while FGF-8b also efficiently activates ‘c’ splice form of FGFR2. No activity could be detected for recombinant or cell expressed FGF-8a. Furthermore, none of the isoforms tested interact efficiently with ‘b’ splice forms of FGFR1-3, or the ‘c’ splice form of FGFR1. These results indicate that the FGF-8b and FGF-8c isoforms, produced by ectodermally derived epithelial cells, interact with mesenchymally expressed fibroblast growth factor receptors. FGF-8b and FGF-8c may therefore provide a mitogenic signal to the underlying mesenchyme during limb and craniofacial development.

Key words: fibroblast growth factor, Fgf8, FGFR, FGF receptors, brain development, limb development, mouse embryogenesis, oncogenes, pharyngeal arches, tyrosine kinase receptors, alternative splicing

SUMMARY

The Fgf8 gene is expressed in developing limb and craniofacial structures, regions known to be important for growth and patterning of the mouse embryo. Although Fgf8 is alternatively spliced to generate at least 7 secreted isoforms that differ only at their mature amino terminus, the biological significance of these multiple isoforms is not known. In this report, we demonstrate that multiple FGF-8 isoforms are present at sites of Fgf8 expression during mouse development. To address the possibility that the FGF-8 isoforms might interact with different fibroblast growth factor receptors, we prepared recombinant FGF-8 protein isoforms. We examined the ability of these proteins to activate alternatively spliced forms of fibroblast growth factor receptors 1-3, and fibroblast growth factor receptor 4. Recombinant FGF-8b and FGF-8c activate the ‘c’ splice form of FGFR3, and FGFR4, while FGF-8b also efficiently activates ‘c’ splice form of FGFR2. No activity could be detected for recombinant or cell expressed FGF-8a. Furthermore, none of the isoforms tested interact efficiently with ‘b’ splice forms of FGFR1-3, or the ‘c’ splice form of FGFR1. These results indicate that the FGF-8b and FGF-8c isoforms, produced by ectodermally derived epithelial cells, interact with mesenchymally expressed fibroblast growth factor receptors. FGF-8b and FGF-8c may therefore provide a mitogenic signal to the underlying mesenchyme during limb and craniofacial development.

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The gene structure of Fgf8 is more complicated than that of most FGF genes. There are at least four exons in Fgf8 that correspond to the usual first exon of other FGF genes. These four upstream exons are alternatively spliced to give potentially eight different FGF-8 protein isoforms that differ at the mature amino termini of the secreted protein isoforms.
The following forward primers: 8A-5′-AACAGTCCCCAGGTAAAGGAGCCTGCGCA-3′; 8B-5′-AAGGATCCAGTTAAGGAGGCTGCGCAGAGGAGCCTGCGCA-3′; 8C-5′-AAGGATCCAGGTAACTGCTGCGCA-3′. For preparation of the amino-terminal tagged isoforms, the following reverse primer was used in PCR: 5′-AAAAGATCTCGGACTCCCGC-3′. For preparation of the carboxy-terminal tagged isoforms, the following reverse primer was used in PCR: 5′-AAAAGATCTCGGACTCCCGC-3′. PCR was performed for 30 cycles at 95°C for 1 minute, 59°C for 1 minute and 75°C for 1 minute, followed by a 10 minute extension at the end of cycling at 75°C. Recombinant Pfu DNA polymerase (Stratagene Inc.) was used with 1× Pfu buffer, 2 mM MgCl2, 0.2 mM dNTPs, 10 mM concentrations of each primer, and 10 ng of target cDNA. The amplified products were purified from agarose gels following electrophoresis, digested with BamHI and either HindIII (for amino-terminal tagged isoforms) or BglII (for carboxy-terminal tagged isoforms), and ligated into appropriately digested pQE30 (for amino-terminal tagged isoforms) or pQE16 (for carboxy-terminal tagged isoforms; Qiagen Inc.).

Plasmid DNA containing the appropriate Fgf8 coding region was obtained following transformation of XL-1 Blue bacteria (Stratagene Inc.) and sequenced to confirm that there were no mutations. Plasmids containing authentic Fgf8 coding regions were expressed in M15 or SG13009 strains of Escherichia coli (Qiagen Inc.). The histidine-tagged recombinant FGF-8 (rFGF-8) isoforms were purified using the denaturing protocol (0.4 M guanidinium hydrochloride, 100 mM sodium phosphate, 10 mM Tris chloride, pH 8.0) of Qiagen and NiNTA agarose chromatography. The denatured purified rFGF-8 isoforms were eluted with 8 M urea, 100 mM sodium phosphate, 10 mM Tris chloride, pH 5.9. The purified rFGF-8 isoforms were renatured by successive dialysis, first against 1 M urea, 100 mM sodium phosphate, 10 mM Tris chloride, 5 mM reduced glutathione, pH 8.0, and then against PBS with 5 mM reduced glutathione. The rFGF-8 isoforms were obtained as a powder by lyophilization, and quantitated by amino acid analysis.

**Antibody preparation and western blotting**

Preparation of the polyclonal anti-mouse FGF-8 antibody that is directed against the FGF-8a isoform, and recognizes all three FGF-8 isoforms tested, has been described previously (MacArthur et al., 1995b). We synthesized a 12 amino acid peptide corresponding to the mature amino terminus of FGF-8b (MacArthur et al., 1995a). This peptide was submitted to Cocalico Biologicals for conjugation to BSA and used to immunize a rabbit. The antibody preparation and western blotting method, as was previously described (Heikinheimo et al., 1994b). For western blot analysis, rFGF-8 isoform proteins and/or conditioned medium from NIH3T3 cells expressing native FGF-8b were separated by 12% SDS-PAGE, electrophoretically transferred to nitrocellulose, blocked with 5% Blotto, and incubated with anti-FGF-8 (0.3 μg/ml) or anti-FGF-8b (0.1 μg/ml) (Harlow and Lane, 1988). A secondary donkey anti-rabbit IgG conjugated with horseradish peroxidase (Amersham) was used at a dilution of 1:3000 with ECL detection, according to manufacturer’s instructions.

**In situ hybridization and immunohistochemical analysis of mouse embryos**

Whole-mount mouse embryos and paraffin sections of mouse embryos were prepared as previously described (Heikinheimo et al., 1994b). For in situ hybridization, paraformaldehyde-fixed whole-mount embryos, were hybridized to sense or anti-sense Fgf8 riboprobes (189 nt, derived from the unique regions of exon 1B; Fig. 1) labeled with digoxigenin-UTP, and detected with anti-digoxigenin antibodies coupled to alkaline phosphatase, as previously described (Heikinheimo et al., 1994b). Paraffin sections of embryos were hybridized with riboprobes labeled with 32P[UTP (ICN), and hybridization was detected by dark-field microscopy (Heikinheimo et al., 1994b).

**MATERIALS AND METHODS**

**Mouse stocks**

Mouse embryos were obtained by mating B6SJLF1/J mice (Jackson labs, ME). Noon on the day the copulation plug was found was considered to be 0.5 days post coitum (E0.5). Precise staging of embryos was performed according to Kaufman’s Atlas of Mouse Development (Kaufman, 1992).

**Preparation of recombinant FGF-8 isoforms**

We have previously cloned Fgf8 cDNAs from murine testis by reverse transcription-polymerase chain reaction methods that code for FGF-8a, FGF-8b, and FGF-8c (MacArthur et al., 1995a). The coding regions for the mature FGF-8 isoforms (i.e., lacking the initiator methionine and signal peptide) were obtained by PCR methods with the following forward primers: 8A-5′-AACAGTCCCCAGGTAAAGGAGCCTGCGCA-3′; 8B-5′-AAGGATCCAGTTAAGGAGGCTGCGCAGAGGAGCCTGCGCA-3′; 8C-5′-AAGGATCCAGGTAACTGCTGCGCA-3′. For preparation of the amino-terminal tagged isoforms, the following reverse primer was used in PCR: 5′-AAAAGATCTCGGACTCCCGC-3′. For preparation of the carboxy-terminal tagged isoforms, the following reverse primer was used in PCR: 5′-AAAAGATCTCGGACTCCCGC-3′. PCR was performed for 30 cycles at 95°C for 1 minute, 59°C for 1 minute and 75°C for 1 minute, followed by a 10 minute extension at the end of cycling at 75°C. Recombinant Pfu DNA polymerase (Stratagene Inc.) was used with 1× Pfu buffer, 2 mM MgCl2, 0.2 mM dNTPs, 10 mM concentrations of each primer, and 10 ng of target cDNA. The amplified products were purified from agarose gels following electrophoresis, digested with BamHI and either HindIII (for amino-terminal tagged isoforms) or BglII (for carboxy-terminal tagged isoforms), and ligated into appropriately digested pQE30 (for amino-terminal tagged isoforms) or pQE16 (for carboxy-terminal tagged isoforms; Qiagen Inc.).

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For western blot analysis, rFGF-8 isoform proteins and/or conditioned medium from NIH3T3 cells expressing native FGF-8b were separated by 12% SDS-PAGE, electrophoretically transferred to nitrocellulose, blocked with 5% Blotto, and incubated with anti-FGF-8 (0.3 μg/ml) or anti-FGF-8b (0.1 μg/ml) (Harlow and Lane, 1988). A secondary donkey anti-rabbit IgG conjugated with horseradish peroxidase (Amersham) was used at a dilution of 1:3000 with ECL detection, according to manufacturer’s instructions.

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Immunohistochemical analyses of whole-mount embryos, and subsequent sectioning of the stained, paraffin-embedded, whole mount embryos, were performed as described (Heikinheimo et al., 1994a). The primary antibody was the anti-mouse FGF-8b (described above) at a concentration of 0.1 μg/ml, and the secondary antibody was an alkaline phosphatase-conjugated goat anti-rabbit IgG used at a 1:200 dilution (Boehringer-Mannheim). Negative controls substituted rabbit pre-immune sera for the primary antibody.

**RNase protection assays**

Total RNA was prepared from dissected limb buds by the acid-phenol method (Chomczynski and Sacchi, 1987). The RNase protection assays were performed as described, using the 5’ anti-sense riboprobes for FGF-8a and FGF-8b (Heikinheimo et al., 1994b).

**Ligand iodination**

FGF-1 was labeled by the Chloramine T method as previously described (Burrus and Olwin, 1989). Briefly, 1-2 μg of FGF-1 was incubated with 1 mCi Na125I (Amersham Inc.) in the presence of 43 μg/ml Chloramine T (Eastman Kodak Inc.), 143 mM Hepes, pH 7.4, in a volume of 70 μl for 2 minutes at room temperature. 100 μl of 20 mM dithiothreitol was then added for 10 minutes. The labeled growth factor was then applied to a heparin agarose column (200 μl bed volume) which had been pre-washed with 20 mM Hepes, pH 7.4, 0.2% BSA, 0.4 M NaCl and washed with 3-5 ml of the same buffer. Ligand was eluted with 20 mM Hepes, pH 7.4, 0.2% BSA, and 3 M NaCl. Labeled growth factor was stored frozen at −70°C for up to 3 weeks.

**Binding assays**

Soluble FGFRs were made in COS cells as previously described (Chellaiah et al., 1994). Binding components were added at 4°C in the following order: DMEM/0.1% BSA, 30 μl of a 2x slurry of anti-AP monoclonal antibodies coupled to Sepharose (Flanagan and Leder, 1990; Ornitz et al., 1992), heparin, FGFR-AP conditioned media (0.3 OD units/minute; Flanagan and Leder, 1990; Ornitz and Leder, 1992) and 125I-lFGF (20-50,000 c.p.m.) in a volume of 250 μl. The reaction was then rotated for 2-3 hours at 4°C. Bound receptor and FGF were recovered by centrifuging (4 seconds at 12000 rpm [4000 g], 4°C in a microcentrifuge), and washed 2 times with 750 μl ice cold PBS. 125I-FGF binding was determined by counting tubes directly in a gamma counter (Beckman Inc.).

**Mitogenic assays**

Full length cDNAs coding for the three immunoglobulin-like domain forms of FGFR1b, 1c, 2b, and 2c were cloned into the expression vector, MIRB. MIRB contains the Moloney murine leukemia virus LTR, unique EcoRI, BamHI and SpeI sites, followed by the IRES-NEO (Ghattas et al., 1991) gene in the Bluescript KS plasmid (described by Chellaiah et al., 1994).

The plasmid pSVFGFR1 IIC was provided by S. Werner (Werner et al., 1992). A 2.9 kb BamHI-SpeI fragment was cloned into the corresponding sites of MIRB. FGFR1 IIC (Yayon et al., 1991) was cloned as a 3.2 kb EcoRI fragment into MIRB by converting a 3’ Asp718 site into an EcoRI site and then excising with EcoRI. FGFR2 IICb (Dell and Williams, 1992) was cloned as a 2.9 kb BamHI fragment into MIRB by converting a 5’ Asp 718 site into a BamHI site and then excising with BamHI. FGFR2 IICc (Mansukhani et al., 1992) was cloned into MIRB as a 3.6 kb SpeI fragment by converting unique NarI and XbaI sites into SpeI sites.

The FGFR3 cDNAs were re-engineered to enhance signaling in BaF3 cells by constructing chimeric cDNAs encoding the extracellular region of FGFR3 fused to the cDNA encoding the tyrosine kinase domain of FGFR1 (referred to as FGFR 31). FGFR 31c has the extracellular region from FGFR3c and the transmembrane domain and tyrosine kinase domain from FGFR1. FGFR 31b has the extracellular region and transmembrane domain derived from FGFR3b (Chellaiah et al., 1994) and the tyrosine kinase domain derived from FGFR1. The details of these chimeric receptors will be described elsewhere. BaF3 cells expressing the two-immunoglobulin-like domain form of FGFR4 were provided by M. Goldfarb (Wang et al., 1994).

MIRB-FGFR plasmids were transfected into BaF3 cells and selected in the presence of 600 μg/ml G418. Individual clonal cell lines were isolated by limited dilution and screened for responsiveness to FGF-1. These cell lines were used in quantitative proliferation assays, measuring [3H]thymidine incorporation into DNA, as previously described (Chellaiah et al., 1994; Ornitz and Leder, 1992; Ornitz et al., 1992). Recombinant FGF-1 (provided by K. Thomas, Merck) was used as a positive control in each experiment. FGF-1 is the only FGF ligand that can activate all FGF receptors.

**RESULTS**

**Location of FGF-8 Isoforms**

Fgf8 contains at least 6 coding exons. The first four exons correspond to the first exon of most other FGF genes (Fig. 1).
Alternative splicing of the four upstream exons results potentially in eight FGF-8 isoforms, seven of which have been identified (Tanaka et al., 1992; Crossley and Martin, 1995; MacArthur et al., 1995a). Prior studies of the expression pattern of \( \text{Fgf8} \) in mouse development used in situ hybridization probes that detect RNA for all the FGF-8 isoforms (Heikinheimo et al., 1994b; Ohuchi et al., 1994; Crossley and Martin, 1995). RNAs specific for the FGF-8a and FGF-8b isoforms were identified by RNase protection assays (Heikinheimo et al., 1994b). However, it is unknown whether there is any temporal or spatial regulation of \( \text{Fgf8} \) splicing. We therefore examined the

Fig. 2. Two polyclonal antibodies to FGF-8 isoforms recognize different rFGF-8 isoforms. Left Panel: 10 ng of rFGF-8a, rFGF-8b, and rFGF-8c were subjected to SDS-PAGE, electrophoretic transfer, and western blotting with an affinity-purified polyclonal rabbit anti-mouse FGF-8 directed against the common carboxyl region of FGF-8 that should recognize all FGF-8 isoforms (MacArthur et al., 1995b). The antibody recognizes all three recombinant FGF-8 isoforms. Center Panel: The blot was stripped and reprobed with a polyclonal rabbit anti-mouse FGF-8b antibody directed against the unique 12 amino acids at the mature amino terminus of FGF-8b. This polyclonal antibody theoretically might interact with FGF-8b, FGF-8d, FGF-8f, and FGF-8h. The antibody recognizes the rFGF-8b isoform. Right Panel: The blot was stripped and reprobed with rabbit pre-immune sera. The left and right panels have been published previously, and are presented here with the permission of the journal of original publication (MacArthur et al., 1995b).

Fig. 3. FGF-8 isoforms recognized by anti-mouse FGF-8b are present in the branchial arches (ba), olfactory pit (op), and apical ectodermal ridge of the limb buds (lb). (A) Whole-mount in situ immunohistochemical staining of an E10.5 mouse embryo, using the polyclonal anti-mouse FGF-8b antibody. (B) Whole-mount in situ immunohistochemical staining of an E10.5 mouse embryo, using rabbit pre-immune sera.

Fig. 4. FGF-8 isoforms recognized by anti-mouse FGF-8b are found on the surfaces of the branchial arches, apical ectodermal ridges of the limb buds, and neuroectodermal regions of the infundibulum. Embryos in Fig. 3 were sectioned for histologic examination. (A,C,D) Polyclonal anti-mouse FGF-8b antibody. (B) Pre-immune sera. (A,B) Section of an E10.5 mouse embryo through the first branchial arches (ba). (C) Section of limb bud (lb) from an E11.5 mouse embryo. (D) Section of the infundibulum (inf) from E11.5 mouse embryo.
temporal and spatial locations of different FGF-8 isoforms by in situ hybridization, by immunohistochemistry and by RNase protection assays of RNA from dissected mouse embryos. As many isoforms share the various combinations of the first four exons, probes or antibodies unique to any particular FGF-8 isoform are not possible (Fig. 1). As there is nothing unique about FGF-8a (Fig. 1), its location can only be examined by analysis of dissected embryo parts.

To elucidate the temporal and spatial location of the FGF-8 isoforms during mouse development, we synthesized a 12 amino acid peptide corresponding to the mature amino terminus of FGF-8b (MacArthur et al., 1995a), of which 11 of the amino acids are encoded by the 5′ portion of exon 1D (Fig. 1). A polyclonal antibody (anti-mouse FGF-8b) directed against this peptide was prepared from rabbits, and recognizes the rFGF-8b isoform, but not the rFGF-8a or rFGF-8c isoforms, in western blot analysis (Fig. 2). A polyclonal antibody directed against rFGF-8a (anti-mouse FGF-8; MacArthur et al., 1995b) recognizes all three rFGF-8 isoforms, while pre-immune sera does not recognize any of the rFGF-8 isoforms in western blots (Fig. 2). The 12 amino acid peptide used to immunize the rabbits is also present in FGF-8f, and 11 of the 12 amino acids in the peptide are present in FGF-8d and FGF-8h (Fig. 1), although the peptide is present at the extreme amino terminus only in the FGF-8b isoform. Using anti-mouse FGF-8b in immunohistochemistry analyses of whole-mount embryos, we find FGF-8 isoforms containing this peptide in the first branchial arches, nasal pits, and AER of the forelimb in E10.5 embryos (data not shown). The pericardial expression observed in this embryo (Fig. 3A) is not consis-

Fig. 5. RNA for FGF-8 isoforms containing sequences encoded by exon 1B is present in the developing central nervous system, branchial arches and limb buds. In situ hybridizations using an anti-sense FGF-8-exon 1B probe of 200 bp in length (A-D,F), or a sense FGF-8-exon 1B probe of 200 bp in length (E). The riboprobes were labeled with [33P]UTP. The antisense probe is predicted to hybridize to RNA for FGF-8c, FGF-8d, FGF-8g, and FGF-8h. (A) Bright-field view of sagittal section of E11.5 mouse embryo. (B) Dark-field view of embryo in A, demonstrating hybridization in the diencephalon (d), mesencephalon-metencephalon junction (m), and branchial arches (ba). (C) Magnified view of box in B, demonstrating hybridization in the infundibulum, maxillary component of the first branchial arch and posterior diencephalon. (D) Dark-field view of E12.5 limb bud, demonstrating ectodermal expression. (E) Dark-field view of branchial arches in E11.5 mouse embryo. (F) Dark-field view of branchial arches and heart in E14.5 mouse embryo.
tently seen (data not shown). RNA for Fgf8 was not found in this pericardial region at E10.5 (Heikinheimo et al., 1994b; Ohuchi et al., 1994; Crossley and Martin, 1995), although the protein produced by branchial arch and/or nasal pit ectoderm may collect along the pericardial surface.

Following staining, the embryos were fixed in paraffin, and sections were prepared for histology (Heikinheimo et al., 1994a). FGF-8 isoform(s) recognized by anti-mouse FGF-8b were detected in surface areas of the first branchial arches (Fig. 4A,B), limb AER (Fig. 4C), and the neuroepithelium of the infundibulum (Fig. 4D). FGF-8 isoform(s) detected by anti-mouse FGF-8b were found also in the neuroepithelium of the telencephalon, diencephalon, and midbrain/hindbrain junction (data not shown). This antibody did not detect any temporal or spatial difference in location of the FGF-8 isoform(s), when compared to prior results using riboprobes that detect RNAs for all of the FGF-8 isoforms (Heikinheimo et al., 1994b; Ohuchi et al., 1994; Crossley and Martin, 1995).

Most of the first four exons are too small to make riboprobes of sufficient sensitivity for in situ analysis. However, an antisense riboprobe transcribed from the 3' portion of exon 1B (Fig. 1) was successfully used for in situ hybridization. This sequence is contained in RNA coding for FGF-8c, FGF-8d, FGF-8g, and/or FGF-8h. This antisense riboprobe was hybridized to mouse embryo sections (Fig. 5). RNAs for FGF-8c, FGF-8d, FGF-8g, and/or FGF-8h were detected in the germinal neuroepithelium of the telencephalon, diencephalon, mesencephalon-metencephalon junction, and infundibulum (Fig. 5A-C), the surface ectoderm of the branchial arches (Fig. 5A-C), and the limb AER (Fig. 5D). The hybridization pattern for this exon 1B riboprobe is identical to our results with a full length riboprobe that detected RNA for all FGF-8 isoforms (Heikinheimo et al., 1994b).

Because FGF-8a cannot be detected with a specific in situ hybridization probe or polyclonal antibody (Fig. 1), we dissected limb buds from E10.5 embryos and prepared total RNA for RNase protection analysis. E10.5 limb bud RNA was hybridized to 5' cDNA riboprobes corresponding to FGF-8a and FGF-8b (Fig. 6). 189 nt of the 286 nt anti-sense FGF-8a probe is protected by RNA for FGF-8a (Heikinheimo et al., 1994b). For the 317 nt anti-sense FGF-8b probe, 222 nt is protected by RNA for FGF-8a (Heikinheimo et al., 1994b). The hybridization pattern for this exon 1B riboprobe is identical to our results with a full length riboprobe that detected RNA for all FGF-8 isoforms (Heikinheimo et al., 1994b).

Mitogenic assays

Our preliminary work with NIH3T3 cells indicates that FGF-8 isoforms possess different potencies with respect to morphological transformation (MacArthur et al., 1995b). We hypothesized that these differences in ability to transform NIH3T3 cells were related to different affinities of FGF-8 isoforms for FGFRs. However, NIH3T3 cells express multiple FGFRs, thus complicating the analysis. We prepared rFGF-8a, rFGF-8b and rFGF-8c isoforms with a six-histidine tag on the carboxyl terminus, since this region was identical in all of the isoforms. These rFGF-8 isoforms were recognized by the polyclonal antibody, anti-mouse FGF-8 (Fig. 2), and the rFGF-8b isoform was recognized specifically by the polyclonal antibody, anti-mouse FGF-8b (Fig. 2). The rFGF-8 isoforms, when applied to culture medium for NIH3T3 cells, result in morphologically altered cells that are similar to cells transfected with the corresponding cDNA (MacArthur et al., 1995b).

To determine the receptor specificity of rFGF-8 isoforms we have developed mitogenic assays that are specific for individual splice forms of FGF receptors 1-3 (Ornitz and Leder, 1992; Ornitz et al., 1992; Chellaiah et al., 1994; see Materials and methods), and FGFR4 (Wang et al., 1994). These assays detect the ability of a preparation of FGF to activate a known splice version of a FGFR and induce a mitogenic response in transfected BaF3 cells expressing the FGFR (measured by 

Fig. 6. RNAs coding for FGF-8a, FGF-8b, and other FGF-8 isoforms are present in developing mouse limbs. Limb buds from E10.5 mouse embryos were dissected and RNA was prepared from the limb buds. The RNAs were analyzed for the presence of species coding for FGF-8 isoforms by ribonuclease protection assay. (A) Ribonuclease protection assay using a 286 nt anti-sense FGF-8a probe. RNA for FGF-8a protects 189 nt. (B) Ribonuclease protection assay using a 317 nt anti-sense FGF-8b probe. RNA for FGF-8b protects 222 nt, and RNAs for the other FGF-8 isoforms protect 153-157 nt. The sizes (nt) of the protected fragments are shown at the left of the figures, and the protected fragments are indicated by arrows at the right of the figures. P, undigested probe; Y, Yeast tRNA; E10, RNA from E10.5 embryos.
FGF-8 isoforms activate FGFR2c, 3c and 4 isoforms activate FGFR2c, 3c and 4 of the FGFRs at concentrations up to 5-10 nM (Fig. 7). Both FGF-8b and FGF-8c activate FGFR4 (data not shown).

FGF-1 activates all FGFRs tested (Fig. 7 and data not shown). To compare the activity of the various FGF-8 isoforms with the splice forms of FGFR1-3, and FGFR4, we have normalized the data in Fig. 7 to that of FGF-1. Relative mitogenic activity was calculated at two points on each curve (312 and 1250 pM for FGFR 1c and 3b, and 625 and 2500 pM for FGFR 1b, 2b, 2c, 3c, and 4Δ). Each pair of points was then averaged, normalized to that of FGF-1 (100%) and plotted. FGFRs 1-3 are three Ig-like domain forms, FGFR4Δ is a 2 Ig-like domain receptor. FGF-1 (■), rFGF-8a ( ), rFGF-8b ( ), rFGF-8c ( ) .

In addition to the alternative mature amino-terminal coding sequences, the FGF-8 isoforms differ in their ability to be glycosylated on asparagine. The FGF-8a and FGF-8e isoforms have no glycosylation sites, the FGF-8b, FGF-8c, FGF-8f, and FGF-8g isoforms have one glycosylation site, and the FGF-8d and FGF-8h isoforms have two glycosylation sites (Tanaka et al., 1992; Crossley and Martin, 1995; MacArthur et al., 1995a). The glycosylation sites for FGF-8b and FGF-8c are known to be used (Tanaka et al., 1992), but whether they are necessary for activity is unknown. The rFGF-8 isoforms, being bacterially produced, are not glycosylated. To test whether the histidine tag and/or lack of glycosylation might alter the ability of the rFGF-8 isoforms to bind to the FGFRs, we repeated some of the mitogenic assays using conditioned medium from NIH3T3 cells stably expressing RNA for the FGF-8b isoform (MacArthur et al., 1995b). Conditioned medium containing native FGF-8b, was quantitated by western blotting with the anti-FGF-8b polyclonal antibody (Fig. 9A), and the concentration of native FGF-8b was found to be approximately 1 mg/ml. Native FGF-8b stimulated mitogenesis from BaF3 cells expressing FGFR3c, but not FGFR1c (Fig. 9B). At very high concentrations (2 μM) native FGF-8b also activates BaF3 cells expressing FGFR1c (data not shown). These results with native FGF-8b (Fig. 9) agree qualitatively with the results obtained...
with rFGF-8b (Fig. 7), but the rFGF-8b is considerably more active than the native FGF-8b preparation. Possible explanations for the decreased activity of the native FGF-8b relative to the rFGF-8b preparation include the possible oxidation of cysteines of the native FGF-8b resulting in polymerization that results in inactivation of much of the native protein, or inhibition and/or competition with other FGFs (i.e., FGF-7) present in the conditioned medium. Regardless of the mechanism of the decreased activity in the native FGF-8b preparation, these results suggest that the carboxy-terminal histidine tags and the lack of glycosylation in the bacterially produced rFGF-8 isoforms do not inhibit their ability to interact with FGFRs.

**DISCUSSION**

Based on the complexity of the Fgf8 gene, resulting in at least seven FGF-8 isoforms, we hypothesize that the FGF-8 isoforms have distinct functions and binding specificities. The data presented here suggest that rFGF-8b is a potential physiological ligand for FGFR3c (and FGFR4 and FGFR2c), but not FGFR1c.
isoforms possess different functions during mouse development. Preliminary evidence indicates that the FGF-8 isoforms behave differently in at least one biological context, namely NIH3T3 cell transformation (MacArthur et al., 1995b). Although the tools for analyzing the temporal and spatial locations of a unique FGF-8 isoform are limited by the similarity of the isoforms (Fig. 1), we have shown that at any particular temporal and spatial location of Fgf8 expression during mouse development, several FGF-8 isoforms are present (Figs 3-6, and data not shown). We further demonstrate that three of the FGF-8 isoforms possess different abilities to activate different splice forms of FGFR1-3, and FGFR4, in vitro (Figs 7-9), and that rFGF-8b binds FGFR3c, but not FGFR1c (Fig. 10). Our results support the hypothesis that these different FGF-8 isoforms serve specific roles during mouse development, based on their ability to bind to and activate mesenchemally expressed ‘c’ splice forms of specific FGFRs. Our results implicate FGF-8b/FGFR2c, FGF-8b/FGFR3c, FGF-8b/FGFR4, FGF-8c/FGFR3c as functional ligand/receptor pairs, assuming that these pairs can be co-localized during development.

The expression patterns of several of the FGFRs are known (Orr-Urtreger et al., 1991; Stark et al., 1991; Yamaguchi et al., 1993; Peters et al., 1993). FGFR1 expression (no distinction between FGFR1c and FGFR1b) was found in mesenchymal regions of the E9.5-E12.5 mouse limb, face and gut, in the germinal epithelium of the developing central nervous system (E9.5-E12.5), and later in neuronal populations in the central nervous system. FGFR2c was also localized to mesenchymal areas of the face, limbs and gut, as well as germinal epithelium of the early developing central nervous system. However, FGFR2b was localized to surface epithelium of the developing face, limbs, and gut. FGFR3 (no distinction between FGFR3b and FGFR3c) was localized to the germinal epithelium of the early central nervous system, glial cells of the more developed central nervous system, the cartilage of developing bone, the lens of the eye, and the sensory epithelium in the developing cochlea. FGFR4 was found in endodermal and myotome derived structures of the E8.5-E16.5 mouse embryo.

The expression patterns of Fgfr8 (Heikinheimo et al., 1994b; Ouichi et al., 1994; Crossley and Martin, 1995) suggest that FGF-8 isoforms may be important regulators of various aspects of mammalian development. In the developing limb bud FGF-8 is expressed in the AER. This expression pattern suggests that FGF-8 isoforms function as a signal that is responsible for the induction of the limb bud (Orr-Urtreger et al., 1991; Peters et al., 1992; Orr-Urtreger et al., 1993; Ohuchi et al., 1994; Crossley and Martin, 1995) and our results indicating that rFGF-8b activates FGFR2c (Fig. 7D), suggest that this interaction is an important early induction event in the formation of the vertebrate limb. Which FGF-8 isoforms are involved in vertebrate limb development remains to be determined.

Although Fgfr8 expression as assessed by in situ hybridization is low to non-existent in the limbs after E13.5 (Heikinheimo et al., 1994b; Ouichi et al., 1994; Crossley and Martin, 1995), it is possible that FGF-8b and/or FGF-8c protein may still be present and activate FGFR3c in pre-cartilaginous condensations of the developing bone at E13.5-E14.5 (Peters et al., 1993).

This same FGF-8b/FGFR2c interaction may also be important in craniofacial development. Our results show that the FGF-8b isoform is present in the surface ectoderm of the branchial arches and nasal pits (Figs 3A and 4A). FGFR2c is known to be present in the mesenchyme of the branchial arches, and later in the developing frontal bones (Orr-Urtreger et al., 1991, 1993; Peters et al., 1992). Hence, it is likely that this same ligand/receptor pair is responsible in part for the outgrowth and patterning of the face.

In the central nervous system, our results suggest that FGF-8b (Fig. 4D) and FGF-8c (Fig. 5) are present in germinal neuroepithelial locations of the telencephalon, diencephalon, mesencephalon-metencephalon junction (isthmus) and neuro-hypophysis (infundibulum). FGFR1, FGFR2c, and FGFR3 are present in these areas as well (Orr-Urtreger et al., 1991; Peters et al., 1992; Orr-Urtreger et al., 1993), suggesting that FGF-8b/FGFR2c, FGF-8b/FGFR3c and FGF-8c/FGFR3c may be ligand/receptor pairs important in the outgrowth and patterning of the telencephalon, diencephalon and mesencephalon-metencephalon junction (isthmus), and pituitary gland.

Fgf8 expression was observed at E14.5 in the developing labyrinth of the ear (Crossley and Martin, 1995). FGFR3 is detected in the differentiating cochlea at E16.5, but not at E14.5 (Peters et al., 1993), so a FGF-8 isoform/FGFR3 ligand/receptor interaction is unlikely to be physiologically relevant. However, FGFR2c and FGFR2b are both present in the cochlear endothelium of E14.5 embryos, indicating that perhaps the FGF-8b/FGFR2c ligand/receptor interaction may be relevant for early inner ear development as well. Fgf8 expression was also observed in the developing renal system at E14.5 (Crossley and Martin, 1995), which overlap with expression patterns of FGFR4 (Stark et al., 1991). Thus, FGF-8b and/or FGF-8c/FGFR4 interactions may be important for the development of kidneys.

The possible role of FGF-8 in craniofacial development suggests that FGF8 may also be involved in several human craniosynostosis syndromes. Apert syndrome (Wilkie et al., 1995), and Crouzon and Jackson-Weiss syndromes (Reardon et al., 1994; Jabs et al., 1994), are all examples of FGFR2 mutations in the extracellular domains that may alter ligand binding and/or receptor activation. In the case of Crouzon syndrome, only 40-50% of the families have identified
mutations in FGFR2, although all cases are thought to map to human chromosome 10q25-26 (Reardon et al., 1994; Jabs et al., 1994). Recently, the human gene encoding FGF8 has been localized to 10q25-26 (White et al., 1995). The co-localization of the FGF8 and FGFR2 genes to 10q25-26, the overlap in expression patterns and the ability of FGF-8b to activate FGFR2c suggests that some cases of Crouzon syndrome may be due to mutations in the FGF8 gene.

We were unable to find any FGFR that interacted with rFGF-8a at the concentrations of rFGF-8a tested (Fig. 7). In contrast, NIH3T3 cells producing FGF-8a following transfection of the cDNA displayed weak morphological transformation (MacArthur et al., 1995b). The explanation for this apparent contradiction is likely related to the very high expression levels obtained following transfection of NIH3T3 cells (Fig. 9A). At tested concentrations (<10 nM), it appears that rFGF-8a has no activity for the FGFRs tested. FGF-8a is not predicted to be glycosylated (MacArthur et al., 1995a; Crossley and Martin, 1995), so the lack of glycosylation in the bacterially produced rFGF-8a isoform should not be the reason for the lack of activity. Recombinant amino-truncated isoforms of FGF-4 and FGF-7 exist (Bellosta et al., 1993; Ron et al., 1993). The truncated FGF-4 is more active than wild-type FGF-4 (Bellosta et al., 1993), while the truncated FGF-7 was able to bind receptor equally with wild-type FGF-7, but was unable to transmit a mitogenic signal (Ron et al., 1993). We have tested the ability of rFGF-8a to inhibit the morphological transformation of NIH3T3 cells by rFGF-8b, however we have observed no effect (C. A. MacArthur, unpublished results). Whether FGF-8a interacts with an unknown FGFR remains to be determined.

Prior work implicates a mutant FGFR1c as the FGF-8 receptor (Sato et al., 1993; Kouhara et al., 1994). The following mutations were described: (1) a 12 hydrophobic amino acid insertion near the signal peptide, thought to be related to alternative splicing; (2) a two amino acid deletion following residue N147; and (3) a H820R substitution at the extreme carboxyl terminus. None of these mutations are located in the sequences that code for the second and third immunoglobulin domains, domains that are thought to be involved in FGF ligand binding (Johnson and Williams, 1993). The functional consequences of the described mutations are not clear. Furthermore our FGFR1 cDNA also lacks the two arginine residues following N147. The FGF-8 protein used in these studies was partially purified from SC-3 cells (Kouhara et al., 1994), and hence was not quantitated. Since SC-3 mammary carcinoma cells make both FGF-8b and FGF-8c (Tanaka et al., 1992), it is not clear which FGF-8 isoform was actually tested. Our results indicate that rFGF-8b and rFGF-8c (Figs. 7B,8), and native FGF-8b (Fig. 9C) interact poorly with FGFR1c at concentrations less than 100 nM. At very high concentrations of native FGF-8b (2 μM), we see some activation of FGFR1c (data not shown), in agreement with prior work (Kouhara et al., 1994). We hypothesize that SC-3 cells under androgen stimulation produce large amounts of FGF-8 protein, comparable to that produced in our NIH3T3 cell line (Fig. 9A). Therefore, the observed ‘binding’ of FGF-8 from SC-3 cells to the mutated FGFR1 in SC-3 cells (Kouhara et al., 1994) is probably due to the enormous amount of FGF-8 present, rather than any inherent affinity of FGF-8b or FGF-8c for the mutated FGFR1c.

The analysis of single FGF-8 isoforms that activate single FGFRs suggest a physiological role in normal development. However, it is also possible that heterodimerization of FGF-8 isoforms and/or FGFRs may occur and may be important for FGF signaling during mouse development (Shi et al., 1993). Heterodimerization of ligands or receptors may further affect the specificity of these interactions.

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REFERENCES


Jabs, E. W., Li, X., Scott, A. F., Meyers, G., Chen, W., Eccles, M., Mao, J.-

Fibroblast growth factor (FGF) receptor 3


