Receptor specificity is an essential mechanism governing the activity of fibroblast growth factors (FGF). To begin to understand the developmental role of FGF-9, a glial activating factor, we have cloned and sequenced the murine FGF-9 cDNA and expressed the protein in mammalian cells and in Escherichia coli. We demonstrate that the FGF-9 protein is highly conserved between mouse and human. Receptor specificity was determined by direct binding to soluble and cell surface forms of FGF receptor (FGFR) splice variants and by the mitogenic activity on cells, which express unique FGF receptor splice variants. Our data demonstrate that FGF-9 efficiently activates the "c" splice forms of FGFR2 and FGFR3, receptors expressed in potential target cells for FGF-9. Significantly, FGF-9 also binds to and activates the "b" splice form of FGFR3, thus becoming the first FGF ligand besides FGF-1 to activate this highly specific member of the FGF receptor family.

Expression and Biological Activity of Mouse Fibroblast Growth Factor-9

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The nucleotide sequences reported in this paper have been submitted to the GenBank®/EMBL Data Bank with accession number(s) U33535.

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The abbreviations used are: FGF, fibroblast growth factor; GAF, glial activating factor; FGFR, fibroblast growth factor receptor; PCR, polymerase chain reaction; kb, kilobase(s).

The ninth member of the FGF family and will henceforth be referred to as FGF-9. Using a rat cDNA probe, FGF-9 expression was detected in rat brain and kidney but not in liver, lung, spleen, thymus, testis, heart, or adrenal gland (2). Like FGF-1 and FGF-2, FGF-9 lacks a signal peptide sequence. Nevertheless, FGF-9 is secreted by the glialoma cell line, NMC-G1, and by transfected COS and Chinese hamster ovary cells (2).

FGF's differentially bind to and activate up to four related transmembrane receptors, which in turn mediate a biological response. FGF receptors (FGFRs) are members of the tyrosine kinase receptor superfamily (5). The extracellular region of the FGFR contains two or three immunoglobulin-like (Ig-like) domains that are differentially expressed as a result of alternative splicing (5). Additionally, another alternative splicing event can alter the sequence of the carboxyl-terminal half of Ig-like domain III without altering the reading frame of the remainder of the receptor. These two splice forms, referred to as "b" and "c," occur for FGFRs 1, 2, and 3 but not 4 (6–9). The specificity of FGF ligand-receptor interaction involves the region of the FGFR ectodomain encompassing Ig-like domain II and III and is dependent on the alternative splicing event in Ig-like domain III (8, 10, 11). The proposed sequence of events involved in the activation of FGFRs includes the formation of a complex between FGF, a heparin-like molecule or a heparan sulfate proteoglycan and an FGFR (12–15). The initial binding event is followed by receptor dimerization, autophosphorylation, and the subsequent activation of downstream signaling molecules (16–20).

In this study, the biochemical characteristics of FGF-9 are further elucidated. We have cloned the murine FGF-9 cDNA and demonstrate that it can transform NIH3T3 fibroblasts. We demonstrate that recombining murine FGF-9 requires heparin for optimal receptor activation and that FGF-9 preferentially binds to and activates FGFR2c and FGFR3c. Additionally, we demonstrate that unlike FGFs 2–8, FGF-9 is able to bind to and activate FGFR3b.

EXPERIMENTAL PROCEDURES

Materials—FGF-1 was a gift from K. Thomas (Merck), FGF-2 was a gift from J. Abraham (Scios Nova, Inc.), FGF-7 was a gift from Amgen Inc., and FGF-8b was purified from Escherichia coli as described (21).

Cloning Murine FGF-9—The FGF-9 cDNA was cloned from mouse brain RNA by reverse transcription-polymerase chain reaction (PCR) methods. Brain cDNA was generated from FVB mouse brain RNA using random hexamers and Moloney murine leukemia virus reverse transcriptase. The oligonucleotides used for PCR were derived from conserved sequences in the rat and human cDNA (4). The forward primer, 5′-GAACCTTGGATTGAAAGAAAGACC-3′, generated a HindIII site (in bold type) at the 5′-end of the amplified fragment. The reverse primer, 5′-CAATTCATAAGAACCACC-3′, generated an EcoRI site (in bold type) at the 3′-end of the amplified fragment. PCR was performed for 30 cycles at 94 °C for 1 min, 55 °C for 2 min, and 72 °C for 2 min, followed by a 5-min extension at the end of cycling at 72 °C. The amplified fragment was cloned into a pGEMS T-vector (Promega) and designated pmFGF9. The cloned PCR fragment was sequenced on an
Expression and Isolation of Recombinant FGF-9—The FGF-9 cDNA was cloned into the bacterial expression vector pV1 and was expressed in E. coli strain W3110 (Environ Inc.) according to methods previously described (22, 23). Cell paste from a 5-liter fermentation was suspended in 400 ml of 35 mm Tris-HCl, pH 7.6, plus 0.2 M NaCl. The solution was clarified by washing with 20 column volumes of the same buffer plus 0.5 M NaCl. Murine FGF-9 was eluted with 5 column volumes of the same buffer plus 1.0 M NaCl. The eluted material was concentrated on an Amicon stirred cell, using a YM5 membrane, to a protein concentration of approximately 1.0 mg/ml, diluted with 35 mM Tris-HCl, pH 7.6, to achieve 0.2 M NaCl, and then reconcentrated to approximately 1.0 mg/ml protein. Final purification was achieved by fast protein liquid chromatography using a Mono-Q column (Pharmacia Inc.) containing neonatal bovine serum, 10% conditioned media from E. coli, and 20,000–60,000 cpm of iodinated FGF were incubated with cells in the cell culture media or binding media. Amino-terminal sequencing of the purified recombinant FGF-9 (Tektagen Inc.) showed that the first five amino acids were PLGEV, which correspond to amino acids 3–7 of the predicted translation from the murine FGF-9 cDNA sequence.

Binding Assays—FGF-1 and FGF-9 were labeled by the chloramine-T method as described previously (24). Briefly, 1–2 μg of FGF was incubated with 1 mCi of Na[125]I (Amersham) in the presence of 43 μg/ml chloramine-T (Pharmacia Biotech Inc.) and 0.1% bovine serum albumin, in a volume of 70 μl for 2 min at 23°C. 100 μl of 20 μM diithiothreitol was then added, and the mixture was then incubated for an additional 10 min at 23°C. The labeled growth factor was then applied to a heparinagarose column (200 m, bed volume), which had been prewashed with 20 mM HEPES, pH 7.4, 0.2% bovine serum albumin, and 0.4 mM NaCl. Labeled growth factor was eluted with 20 mM HEPEs, pH 7.4, 0.2% bovine serum albumin, and 3 mM NaCl and stored frozen at −70°C for up to 14 days.

Soluble FGFR (1c, 2b, 3b, and 3c)-alkaline phosphatase fusion proteins were made in COS cells as described previously (8, 18). Binding components were added at 4°C in the following order: Dulbecco's modified Eagle's medium, 10% fetal calf serum, L-glutamine, 1 mM thiols (mercaptoethanol, dithiothreitol), and 20 mM HEPES, pH 7.4, in a volume of 120,000 rpm (4000 g, 4°C in a microcentrifuge), and washed twice with 500 ml of ice-cold phosphate-buffered saline. 125I-FGF binding was determined by counting the washed tubes directly in a γ counter (Beckman). Binding to cell surface FGFR receptors was performed as for the soluble FGF receptors except that anti-alkaline phosphatase-Sepharose and FGFR-alkaline phosphatase monoclonal antibodies coupled to Sepharose (25), 10 μl of 25 μM heparin, 50 μl of FGF-alkaline phosphatase-conditioned media containing specific soluble FGFRs (0.3 optical density units/min) (18), non-iodinated FGF as a competitor, and 125I-FGF (20,000 cpm) in a total volume of 250 μl. The reaction was then gently rotated for 90 min at 4°C. Bound receptor and FGF were recovered by centrifugation (10 s at 12,000 rpm [4000 g, 4°C in a microcentrifuge]), and washed twice with 500 ml of ice-cold phosphate-buffered saline. 125I-FGF binding was determined by counting the washed tubes directly in a γ counter (Beckman). 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or activate this receptor (8). Therefore, to further characterize this binding interaction, we tested the ability of FGF-1, FGF-2, and FGF-9 to compete with the binding of labeled FGF-9 to FGFR3b. Both FGF-1 and FGF-9 can efficiently compete for binding to FGFR3b; however, FGF-2 was only a weak competitor (Fig. 3). This is consistent with the inability of FGF-2 to bind to or activate FGFR3b (8).

FGF-1 binds with high affinity to all known FGFRs (7–10, 26, 28–30). Competition binding experiments demonstrate that FGF-9 cannot compete with labeled FGF-1 for binding to FGFR1c yet does demonstrate an increasing ability to compete with FGF-1 for binding to FGFR2b, FGFR3b, and FGFR3c (Fig. 3c), respectively. These data are consistent with the direct binding experiments in which FGF-9 preferentially binds to FGFR3c and less well to FGFR2b and FGFR3b. The ability of FGF-9 to compete with labeled FGF-1 for receptor binding is poor compared to that of unlabeled FGF-1. However, this observation is consistent with previous results in which FGF-7 could only partially compete with FGF-1 for binding to its primary receptor, FGFR2b (8).

Receptor Activation by Recombinant FGF-9—To further test the biological activity of FGF-9, we assayed its ability to activate the "b" and "c" splice forms of FGFRs 1–3. FGFRs 1b, 1c, 2b, and 2c were expressed in BaF3 cells as full-length receptors. Two additional control ligands were also used, FGF-7 and FGF-8. FGF-7 can only activate FGFR2b (Fig. 4c), while FGF-8 specifically activates FGFR2c and FGFR3c (Fig. 4d and f) (21). Similar to FGF-8, recombinant FGF-9 most efficiently activates FGFR2c and FGFR3c; however, unlike with FGF-8, FGF-9 can also partially activate FGFR1c and FGFR3b (Fig. 4). FGF-9 shows no activity toward cells expressing FGFR1b and FGFR2b. The strong activity toward FGFR3c is consistent with the cell surface and soluble receptor binding data. The weak activity toward FGFR3b is consistent with binding to soluble receptor. However, we do not detect binding to cell surface or soluble FGFR1c even though cells expressing this receptor can activate this receptor (8).
be weakly activated by FGF-9. Conversely, we do detect weak binding to soluble (but not cell surface) FGFR2b even though cells expressing this receptor are not activated by FGF-9. The physiological significance of these activities toward FGFR1c and FGFR2b remain to be defined.

To assess the relative mitogenic activity on individual FGFR splice variants, we normalized the mitogenic data in Fig. 4 to that of FGF-1. The relative mitogenic activity for each ligand ([3H]thymidine incorporation) at concentrations of 312 and 1250 nM was calculated. These values were then averaged and plotted in Fig. 5. This analysis clearly demonstrates that the best receptors for FGF-9 are FGFR2c and FGFR3c. These FGFRs can be activated by FGF-9 with 89 and 96% of the activity of FGF-1, respectively. FGFR3b-expressing cells respond to FGF-9 with 42% of the activity of FGF-1, and FGFR1c-expressing cells respond to FGF-9 with 21% of the activity of FGF-1.

Receptor Activation Specificity of Native FGF-9—To assess the activity of native FGF-9 and to compare it to that of recombinant FGF-9, we transfected NIH3T3 cells with an FGF-9 cDNA expression vector (see "Experimental Procedures"). Transfected cells were either allowed to grow to confluency in a focus-forming assay or selected for expression of the transfected plasmid with the drug G418. Both selection methods result in the morphological transformation of NIH3T3 cells. Foci or selected colonies grew in a disorganized manner, with the majority of cells assuming a spindle-shaped morphology (data not shown). Media conditioned by these cells was then assayed for mitogenic activity on BaF3 cells expressing FGFR1c, FGFR3b, or FGFR3c (Fig. 6). Similar to recombinant FGF-9, FGF-9-expressing NIH3T3 cell-conditioned media activate FGFR3c. FGFR3b without competitor (−) or with 20 nM unlabeled FGF-1, 2, or 9 as indicated. C, relative binding of iodinated FGF-1 to soluble FGFRs 1c, 2b, 3b, and 3c. Shaded bars, no competitor; open bars, competition with 80 nM unlabeled FGF-1; solid bars, competition with 80 nM unlabeled FGF-9. Error bars indicate standard deviation.

FGF-9 Requires Heparin for Optimal Mitogenic Activity—All known members of the FGF family bind heparin with relatively high affinity (Kd ~ 10^{-9} M) (1), and several of the FGFs have been shown to require heparin for optimal biological activity (14, 18, 28). In biological assays, heparin is likely to serve at least two functions: 1) heparin can increase the stability and thus the half-life of FGF molecules and 2) heparin can stabilize and thus increase the half-life of ligand-receptor interaction. Like other members of the FGF family, FGF-9 is also a heparin binding protein. To determine the effect of heparin on the
biological activity of FGF-9, we compared the heparin dependence of FGF-9 mitogenic activity to that of FGF-1 on BaF3 cells expressing FGFR3c (Fig. 7). Both FGF-1 and FGF-9 require heparin for maximal biological activity on FGFR3c-expressing BaF3 cells. Half-maximal activation of FGF-1 is seen at 670 ng/ml heparin, and half-maximal activation of FGF-9 is seen at 185 ng/ml heparin. Compared to FGF-1, FGF-7, and FGF-8, FGF-9 requires lower heparin concentrations for optimal activity. This difference in heparin dependence may reflect a higher affinity for heparin by these two FGFs.

DISCUSSION

FGFs compose a family of growth factors that play key roles in a variety of developmental events. Some FGFs are expressed in adult tissues and may be important for maintaining normal tissue homeostasis. FGFs are also involved in mediating a physiological response to injury (31). Our data indicate that the preferred receptors for FGF-9 are FGFR2c, FGFR3c, and FGFR4. Additionally, the binding and mitogenic data presented here demonstrate that FGF-9 can also bind to and activate FGFR3b. Although the activity of FGF-9 toward FGFR3b is only 42% of that of FGF-1, it is nevertheless significant because no other FGF ligand shows any activity toward this receptor (8).

FGFR2 is expressed in glial cells (32), low grade astrocytomas (33), and oligodendrocytes present in fiber tracts of the central nervous system (34). FGFR2 transcripts have been identified in the germinal epithelium of the developing central nervous system and, later in development, in a diffuse pattern consistent with expression in glial cells (35). FGFR2 is also prominently expressed in epithelial tissues in limb bud, kidney, stomach, and lung (35, 36). FGFR3 mRNA has been localized to the germinal epithelium of the developing central nervous system, to glial cells (later in development), to the sensory epithelium of the cochlea, to proliferating cartilage of developing bone, and to the lens of the eye (37). Although the cellular localization of FGFR3b and FGFR3c is not known, RNase protection studies indicate that both splice forms are expressed in kidney (8). In the case of FGFR2, "b" splice forms are restricted to epithelial tissues, and "c" splice forms are expressed in mesenchymal tissues (10, 38).

The identification of FGFR2 and FGFR3 expression in glial cells and the identification of a functional ligand, FGF-9, expressed in both brain and kidney (2), during development, FGF-9 is expressed at low levels in mid-gestation mouse embryos. Thus, it is likely that FGF-9 plays a role in both developmental events and in normal adult physiology. Important elements controlling the activity of FGFs include tissue- and temporal-specific gene expression and specificity of ligand-receptor interactions. In this study, we have determined the receptor specificity of FGF-9, thus identifying its potential physiologically relevant receptors.

Our data indicate that the preferred receptors for FGF-9 are FGFR2c, FGFR3c, and FGFR4. Additionally, the binding and mitogenic data presented here demonstrate that FGF-9 can also bind to and activate FGFR3b. Although the activity of FGF-9 toward FGFR3b is only 42% of that of FGF-1, it is nevertheless significant because no other FGF ligand shows any activity toward this receptor (8).

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Fig. 5. Relative mitogenic activity of FGFs. Mitogenic activity at concentrations of 312 and 1250 pM for each FGF (see Fig. 4) was normalized to that of FGF-1 and then averaged and plotted. Error bars indicate the average of the standard deviations at the two FGF concentrations used. Solid bars, FGF-1; open bars, FGF-7; hatched bars, FGF-8; shaded bars, FGF-9.

Fig. 6. Relative mitogenic activity of native FGF-9. The relative mitogenic activity of BaF3 cells expressing FGFR1c, FGFR3b, and FGFR3c is shown in the presence of 100 pM FGF-1 (shaded bars), 10 μl of conditioned media from confluent FGF-9-transformed NIH3T3 cells (solid bars), or control NIH3T3 cell-conditioned media (open bars). The data were normalized to that of FGF-1. Error bars indicate standard deviation.

Fig. 7. Heparin dependence of FGF-1 and FGF-9. Mitogenic activity (3H)thymidine incorporation) of FGFR3c-expressing BaF3 cells at varying concentrations of heparin in the presence of 200 pM FGF-1 (circles) and 200 pM FGF-9 (squares) is shown.
count for the formation of the tumor that originally gave rise to this cell line. Additional support for FGF-9 forming physiologically relevant ligand-receptor pairs with FGFR2 and FGFR3 comes from gene expression in kidney where FGF-9 (2), FGFR2c, FGFR3, and FGFR4 are all expressed (30, 37, 39). FGF-9 may therefore play a role in both neural and renal development, tissue homeostasis, and response to injury. Spatial localization of FGF-9, FGFR2, and FGFR3 at the cellular level should help to define the physiological relationship between these signaling molecules.

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