Characterization of Recombinant Human Fibroblast Growth Factor (FGF)-10 Reveals Functional Similarities with Keratinocyte Growth Factor (FGF-7)*

A newly identified member of the fibroblast growth factor (FGF) family, designated FGF-10, is expressed during development and preferentially in adult lung. The predicted FGF-10 protein is most related to keratinocyte growth factor (KGF, or FGF-7). The latter is expressed exclusively by epithelial cells. In order to examine the biological and biochemical properties of human FGF-10, we isolated the cDNA and expressed its encoded protein in bacteria. The recombinant protein (rFGF-10) was a potent mitogen for Balb/MK mouse epidermal keratinocytes with activity through the FGF receptor (FGFR2b) isoform KGFR receptor (KGFR) expressed specifically by epithelial cells. In this concentration range, FGF-10 did not stimulate DNA synthesis in NIH/3T3 mouse fibroblasts. rFGF-10 bound the KGFR with high affinity comparable to that of KGF, and did not bind detectably to either the FGFR1c (Flg) or FGFR2c (Bek) receptor isoforms. The mitogenic activity of FGF-10 could be distinguished from that of KGF by its different sensitivity to heparin and lack of neutralization by a KGF monoclonal antibody. These results indicate that FGF-10 and KGF have similar receptor binding properties and target cell specificities, but are differentially regulated by components of the extracellular matrix.

The fibroblast growth factors (FGFs)1 comprise a family of at least 14 members (1–11). In addition to their ability to stimulate proliferation of a wide variety of cells, FGFs exhibit potent neurotrophic and angiogenic activities (12–15). They also have the capacity to inhibit or maintain a differentiated phenotype in a variety of cells in culture (15–21). These molecules have activities at early and late stages of development and are widely expressed in adult tissues, indicating that they likely play important roles as growth and differentiation factors throughout the life span of the organism. FGFs stimulate cells by interacting with cell surface tyrosine kinase receptors (22, 23). Four closely related receptors (FGFR1–FGFR4) have been identified. FGFR1–FGFR3 genes have been shown to encode multiple isoforms generated by alternative splicing (24), and these isoforms can be critical in determining ligand specificity (25–29). Thus, the relationships between FGF receptors and their ligands are complex. Most FGFs bind more than one receptor (23, 25–32). However, KGF is unique among FGFs in that it interacts only with a specific isoform of FGFR2, designated FGFR2b or KGFR (27, 33), which is expressed exclusively by epithelial cells.

Rat FGF-10 was recently cloned by homology based polymerase chain reaction using degenerate primers to common amino acid sequences in the FGF-3 and FGF-7 coding regions (10). Its mRNA was shown to be preferentially expressed in adult lung and in several discrete regions of the embryo (10, 34). Among the known FGF family members, rat FGF-10 possesses the highest sequence similarity to KGF. Thus, we reasoned that it might have similar biological properties. In order to test this hypothesis, we cloned the human FGF-10 cDNA and produced functional recombinant FGF-10 (rFGF-10) in bacteria for characterization of its physical and biochemical properties.

EXPERIMENTAL PROCEDURES

Isolation of Human FGF-10 cDNA—RNA prepared from different human cell lines and tissues was used as a template for reverse transcription-polymerase chain reaction. RNA was incubated for 60 min at 37 °C in a reaction mixture containing 300 units of Moloney murine leukemia virus reverse transcriptase, 15 units of human placenta RNase inhibitor, and 0.5 μg of random hexadeoxynucleotide primer. The polymerase chain reaction was performed for 35 cycles at 94 °C for 45 s, 55 °C for 45 s, and 72 °C for 2 min in a reaction mixture containing an aliquot of the above cDNA solution, 0.05 unit/μl Taq DNA polymerase, and 3 pmol/μl each of the primers sequences 5′-ATC ATA TGA CTC TTT GTC AGG ACA TG-3′ and 5′-ATG CAT CCA TTA GTC GAC CAC CAT-3′. These corresponded to nucleotides 112–648 of the rat FGF-10 cDNA sequence with additional Ndel and BamHI sites located at their 5′ ends, respectively. An in-frame methionine codon within the Ndel site served as a translation initiation site for recombinant expression in Escherichia coli. Amplified DNA was cloned into the TA cloning vector PCR 2.1 (Invitrogen, Carlsbad, CA), and a number of clones were sequenced to confirm their identity as human FGF-10 cDNA.

Production and Purification of Recombinant Human FGF-10—The Ndel/BamHI fragment of human FGF-10 cDNA was subcloned into the corresponding sites of pET 9c, to allow production of recombinant protein from the T7 610 promoter (35). BL21(DE3) pLYsS cells bearing the recombinant plasmid were grown overnight at 37 °C in Luria-Bertani medium containing 25 μg/ml kanamycin. The culture was diluted 100-fold with Luria-Bertani medium containing 25 μg/ml kanamycin and incubated for 3 h prior to addition of isopropyl-1-thio-β-d-galactopyranoside (1 mM) for 3 h to induce rFGF-10 expression. Cells were collected by centrifugation, and the cell pellet was resuspended in TENG buffer (10 mM Tris-HCl, 200 mM NaCl, 1 mM EDTA, 5 mM MgCl2, pH 7.5) containing protease inhibitors. The cells were lysed by three successive cycles of freezing and thawing, disrupted by sonication, and cleared by centrifugation, and the supernatant was frozen at −70 °C until required.
Purification of recombinant FGF-10 was performed by applying the bacterial supernatant (5 ml) to a heparin-Sepharose column (1-ml bed volume), (Pharmacia Biotech Inc.). The column was washed with 20 bed volumes of 50 mM phosphate buffer, pH 7.2, containing 0.2 M NaCl and then subjected to a stepwise gradient of increasing NaCl concentration. Aliquots from fractions were analyzed by SDS-PAGE, and selected fractions were concentrated 10–20-fold with a Centricon-10 microcentrator (Amicon, Beverly, MA) for use in biological and biochemical analyses.

Mitogenic Assays—Thymidine incorporation into Balb/MK mouse epidermal keratinocytes (36) and NIH/3T3 mouse embryo fibroblasts (37) was performed as described previously (38). Briefly, cells were grown on 96-well microtiter plates precoated with fibronectin. Cells were serum-starved for 24 h, treated with various concentrations of growth factors for 17 h, and incubated with 4 \( \mu \text{Ci/well} \) [\( ^3\text{H} \)]thymidine for 6 h, and [\( ^3\text{H} \)]thymidine incorporation was measured by a liquid scintillation counter. Neutralization experiments utilized monoclonal antibody 1G4, directed against KGF (39). For these experiments, the growth factor and monoclonal antibody were also added simultaneously. For experiments performed in the presence of heparin, the growth factor and heparin were added at the same time.

Growth Factor Iodination and Receptor Binding Assay—KGF and FGF-10 were labeled with Na\(^{125}\)I using the chloramine-T method (40) as described previously (41). The specific activity for each iodinated growth factor was about \( 5 \times 10^5 \) cpm/ng. \(^{125}\)I-Labeled growth factor binding and competition assays were performed using NIH/3T3 cells transfected with either FGFR2b (KGFR), FGFR1c (Flg), or FGFR2c (Bek) as described previously (33, 42).

## RESULTS

### Cloning and Recombinant Bacterial Expression of Human FGF-10—Oligonucleotide primers designed to amplify rat FGF-10 residues Ala\(^{38}\) to Ser\(^{215}\) were also used to amplify the human FGF-10 (Fig. 1A). The amino acid sequence of the isolated human FGF-10 cDNA was identical to that of rat FGF-10 with the exception of an eight-serine residue deletion (96% identity, SIM program) (43). The predicted human FGF-10 product had highest similarity to that of KGF, and the homology of the conserved region (amino acids 60–205 of human FGF-10) between FGF-10 and KGF or FGF-3 was 54 and 47%, respectively (SIM program) (43). The predicted molecular mass of this molecule was 19 kDa.

The human FGF-10 sequence was subcloned into the pET9c plasmid to allow recombinant expression in *E. coli* from the promoter of the highly expressed \( f_\text{10} \) gene of bacteriophage T7 (35). The amplified region corresponded to that encoding the putative mature growth factor following secretion and cleavage of the signal sequence. rFGF-10 was induced and purified as described under “Experimental Procedures.” Analysis of successive fractions of increasing NaCl concentration eluted from the heparin-Sepharose column determined that the majority of rFGF-10 eluted with the 1.0 M NaCl fraction, as determined by...
higher in the lumen of the protein compared with the predicted molecular mass. Amino acid analysis of a sample of protein eluted at 1 M NaCl confirmed that the preparation consisted of substantially pure FGF-10, whose amino terminus was in agreement with that predicted from the sequence.2

Mitogenic Activity—Because the primary structure of FGF-10 was most similar to that of KGF among the known FGF family members, we sought to compare its biological and biochemical properties with those of KGF. To do so, we first examined their mitogenic activities as a function of concentration on Balb/MK keratinocytes, which naturally express the KGFR, and NIH/3T3 fibroblasts, which do not. As shown in Fig. 2, while KGF stimulated DNA synthesis in Balb/MK cells at less than 0.005 nM, stimulation by rFGF-10 required growth factor concentrations of greater than 0.01 nM. Neither growth factor stimulated NIH/3T3 cells at these concentrations. However, rFGF-10 was able to stimulate significant DNA synthesis in these cells at concentrations greater than 5 nM (Fig. 2).

Receptor Binding Properties of FGF-10—FGF-10 appeared to have a target cell specificity similar to that of KGF, which raised the possibility that it might also bind to the KGFR. To explore this further, we examined its ability to compete with KGF for binding to the KGFR. Binding experiments were performed with NIH/3T3 cells overexpressing the KGFR (33). As shown in Fig. 3A, rFGF-10 was able to displace 125I-KGF from the KGFR with only slightly less efficiency than unlabeled KGF. Reciprocal experiments showed that KGF also efficiently competed 125I-FGF-10 binding (Fig. 3B). bFGF, which has a low affinity for the KGFR, did not displace either 125I-KGF or 125I-FGF-10 under the same conditions. To confirm the specific binding of FGF-10 to the KGFR, we examined the binding of FGF-10 to NIH/3T3 cells overexpressing either KGFR, FGFR1c, or FGFR2c (42). Because NIH/3T3 cells have heparan sulfate-type proteoglycans, which are known to exhibit low affinity binding to FGFs, we performed the assay with increasing heparin concentration such that only specific binding of FGF-10 to its high affinity receptor(s) might be detected. As shown in Fig. 3C, only NIH/3T3 cells overexpressing KGFR bound FGF-10 in the presence of heparin. Even though NIH/3T3 cells overexpressing FGFR2c exhibited slightly greater binding of FGF-10 than did control NIH/3T3 cells, we were unable to detect specific competition by unlabeled FGF-10 or bFGF (data not shown). These results confirm that FGF-10 binds the KGFR but does not bind either FGFR1c or FGFR2c with high affinity.

Differential Heparin Effects on FGF-10 and KGF Mitogenic Activities—The activities of FGFs are modulated by heparin (44–46). For example, the biological activities of aFGF are highly dependent on heparin, whereas those of KGF are strongly inhibited (45–47). To characterize heparin effects on FGF-10 mitogenic stimulation of Balb/MK cells, we performed thymidine incorporation assays at different concentrations of heparin (Fig. 4A). Stimulation by heparin of FGF-10 activity was readily observed at low FGF-10 concentrations, whereas heparin was inhibitory to KGF under the same conditions. The stimulatory effects of heparin were most pronounced with aFGF on both Balb/MK and NIH/3T3 cells. Heparin did not increase the sensitivity of NIH/3T3 cells to FGF-10 (Fig. 4A). We performed a more detailed heparin dose response analysis to further define the effects of heparin on FGF-10 mitogenic activity. As shown in Fig. 4B, the addition of heparin at 0.3 µg/ml was optimal for stimulating FGF-10 activity. At this heparin concentration, its activity was comparable to that of KGF in that it stimulated DNA synthesis in Balb/MK cells at picomolar concentrations (Fig. 4C). Recombinant KGF is 5–10-fold more potent than the glycosylated naturally occurring form (41). Thus, these findings strongly imply that recombinant FGF-10 was produced and purified in a fully active form.

To further distinguish FGF-10 biological activity from that of KGF, we performed an antibody inhibition assay on Balb/MK cells using a neutralizing KGF monoclonal antibody (clone 1G4) (39). As shown in Fig. 5, this antibody was able to neutralize the mitogenic activity of KGF in a dose-dependent fashion (90% inhibition at 100 µg/ml of antibody), while FGF-10 and bFGF were not significantly affected even at the highest antibody concentration tested.

DISCUSSION

In the present study, we isolated the cDNA encoding human FGF-10 and characterized the recombinant protein produced in bacteria. Among known FGF family members, FGF-10 is most similar in its predicted sequence and functional properties to KGF. Like KGF, FGF-10 showed preferential mitogenic activity for Balb/MK keratinocytes as compared with NIH/3T3 fi-
FIG. 3. Competition for $^{125}$I-KGF binding (A) or $^{125}$I-FGF-10 binding (B) to NIH/3T3 cells overexpressing the KGF receptor by increasing concentrations of KGF, rFGF-10, and bFGF. Values are the mean of triplicate samples. Where no error bars are shown, the error was less than the symbol size. C, binding of $^{125}$I-FGF-10 to NIH/3T3 cells overexpressing the KGFR, FGFR1c (Flg), or FGFR2c (Bek) receptors as a function of increasing concentration of heparin as described in the “Experimental Procedures.” Values are the mean of triplicate samples.

FIG. 4. Effect of heparin on the stimulation of DNA synthesis in Balb/MK and NIH/3T3 cells by FGF-10, KGF, and aFGF. A, stimulation of $[^{3}H]$thymidine incorporation in Balb/MK and NIH/3T3 cells by FGF-10, KGF, or aFGF as assessed in the presence (1 and 10 ng/ml) or absence of heparin as described under “Experimental Procedures.” B, dose-response curve of heparin effects on FGF-10 mitogenic activity. Balb/MK cells were stimulated with 0.05 nM of rFGF-10 in the presence of varying concentration of heparin as described in the “Experimental Procedures.” Values are the mean of triplicate samples. C, comparison of the mitogenic activities of recombinant FGF-10 and KGF on Balb/MK cells in the presence (0.3 μg/ml) or absence of heparin as described above. Results represent mean values from triplicate samples. Where no bars are shown, the error was less than the symbol size.
Broblasts. By means of binding/competition analysis, we further established that these two growth factors interact with the same high affinity receptor, the KGFR isofrom of FGFR2, which differs from FGFR2 in the second half of the third immunoglobulin loop and is encoded by an alternative exon (33). The ability of KGFR and FGF-10 to bind the KGFR with high affinity distinguishes them from other members of the FGF-10 family (23–25).

Unlike KGF, FGF-10 exhibited significant mitogenic activity on NIH/3T3 cells at high concentration. NIH/3T3 cells do not express the KGFR, and we did not detect specific high affinity binding of FGF-10 to either FGFR1c or FGFR2c, when they were overexpressed by these cells. Thus, FGF-10 mitogenic effects for NIH/3T3 cells at high concentration likely reflect functional interactions with some other FGFR isoform(s).

We observed that heparin exerted differential effects on FGF-10 and KGF. Whereas KGFR mitogenic activity was inhibited by heparin, it was stimulatory to FGF-10. At an optimal concentration of 0.3 μg/ml heparin, the mitogenic effects of FGF-10 were stimulated as much as 5–10-fold, such that it was comparable to that of recombinant KGF. Because both the concentration and composition of heparan sulfate containing proteoglycans on the cell surface or extracellular matrix vary among cell types and tissues, these molecules likely contribute to regulation of the sensitivity and, thus, spectrum of responses to FGF-10 and KGF, which signal through the same receptor, may be mediated in vivo.

While the manuscript of this work was in preparation, Emoto et al. (48) reported the cloning of human FGF-10, whose sequence is identical to that reported here. In their study, recombinant human FGF-10 was found to exhibit mitogenic activity for rat epidermal keratinocytes but not NIH/3T3 cells at concentrations (48) where we also observed no detectable activity. The cloning of mouse FGF-10 and characterization of its processing has also been recently reported (49). It was shown that FGF-10 is glycosylated and remains predominately cell- or extracellular matrix-associated, but can be released into medium by heparin (49). In contrast, KGF, which is also glycosylated, is readily secreted into the medium of producing cells (50). The different secretory properties of these functionally related FGFs may reflect the differences in their respective affinities for proteoglycans.

KGF is expressed by stromal cells and acts as a paracrine mediator of epithelial cell proliferation/differentiation (50–52). Analysis of FGF-10 expression in mouse skin revealed its transcript in dermis but not epidermis, consistent with FGF-10 also acting in a paracrine manner on epithelial cells (49). In addition to being necessary for organogenesis during development, paracrine acting signals originating from the stroma play a crucial role in tissue remodeling and epidermal regeneration following wounding (53, 54). In vivo, KGF expression is dramatically induced during cutaneous wound repair (53, 54). Furthermore, the KGF transcript can be induced by treatment of fibroblasts with proinflammatory cytokines such as interleukin-1 (55, 56). These agents have been shown to up-regulate transcription of the KGF gene, thus providing a mechanistic basis for its induction following wounding. In addition, the demonstration of high levels of KGF expression in chronic inflammatory diseases of the skin (57) and bowel (58) provide further evidence of its up-regulation in an inflammatory environment. Tagashira et al. (59) reported a marked increase in mouse FGF-10 transcript levels following cutaneous wounding. In contrast, Beer et al. (49) reported no induction under similar conditions, nor did these workers observe FGF-10 induction in fibroblasts following stimulation by proinflammatory mediators or serum growth factors. Thus, whether FGF-10 is up-regulated, like KGF, in response to superficial wounding will require further investigation.

KGF knockout mice show no obvious abnormalities in epidermal growth or wound healing (60), and even transforming growth factor-α/KGF double knockout mice have been reported to exhibit unimpaired wound healing (60). Yet, transgenic mice expressing a dominant-negative KGF receptor show epidermal atrophy and delayed wound healing (61). These findings strongly suggest the existence of other KGFR ligands with redundant or overlapping functions to KGF. Our findings that FGF-10 possesses similar biological and biochemical properties to KGF suggest it as a candidate. Studies of FGF-10 knockout and KGF/FGF-10 double knockout mice will be necessary in order to fully determine the respective roles of these two functionally related FGFs in mediating epithelial proliferation, morphogenesis, and differentiation during normal development, and tissue repair.

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REFERENCES

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