

Conservation of Ligand Specificity between the Mammalian and Amphibian Fibroblast Growth Factor Receptors*

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We have previously cloned and sequenced a newt keratinocyte growth factor receptor (KGFR) cDNA which exhibited a unique spatial and temporal expression pattern in the regenerating newt limb. In this report, we further characterize the biochemical and functional properties of this newt KGFR. A stable Chinese hamster ovary transfectant overexpressing the newt KGFR was capable of binding both ¹²⁵I-fibroblast growth factor-1 (FGF-1) and ¹²⁵I-FGF-7 but not ¹²⁵I-FGF-2, indistinguishable from the human KGFR. Scatchard analysis and cross-linking studies further support the conclusion that FGF-1 and FGF-7 are the ligands for the newt KGFR. In addition to their ability to bind to FGFs, both the human and the newt KGFR are also capable of repressing differentiation in mouse MM14 myoblasts. MM14 cells express FGFR1 and are repressed from differentiation by FGF-1, FGF-2, and FGF-4 but not FGF-7. Co-transfection of MM14 cells with either a human or newt KGFR expression construct conferred a response to FGF-7 as determined by a human α -cardiac actin/luciferase reporter construct. The response to FGF-7 was similar to the endogenous FGF response as FGF-7 prevented MM14 myoblasts from undergoing terminal differentiation. Thus, both the human and the newt KGFRs transduce signals similar to those transduced via the endogenous mouse FGFR1. Together these data indicate that this newly isolated newt KGFR is a functional receptor as it binds two FGF family members with high affinity and mediates signaling in skeletal muscle myoblasts. Because the binding pattern of the newt KGFR is similar to the pattern observed for its mammalian counterpart, it emphasizes the strict conservation that this ligand/receptor system has undergone through evolution.

Fibroblast growth factors (FGF)¹ elicit a multitude of different biological responses in a variety of mesodermal and neuroectodermal derived cell types and are implicated in several

physiological and pathological processes (reviewed in Refs. 1 and 2). The distinct biological responses of cells to FGFs thus far appear to be mediated by a family of transmembrane FGF receptors (FGFRs). These include FGFR1/*flg* (3–5), FGFR2/*bek* (4, 6, 7), FGFR3 (8), and FGFR4 (9). The four receptors constitute a subclass of receptor tyrosine kinases that is characterized by three (or two) extracellular immunoglobulin-like domains (Ig domains), a membrane spanning region, and a cytoplasmic portion that contains a tyrosine kinase domain (10). FGFRs 1–3, but not FGFR4, are subject to a high degree of alternative splicing of their primary transcripts that results in a multitude of combinatorial splice variants (reviewed in Ref. 11). Alternative splicing within the tyrosine kinase domain of FGFR1 can result in a kinase-defective molecule. Upon ligand binding this kinase-defective variant oligomerizes with a kinase-containing FGFR resulting in a heterodimer complex incapable of phosphorylation and activation of phospholipase C γ (12). However, the majority of alternative splicing events occur in the extracellular portion of the receptor. For example, cDNAs for FGFR1 and FGFR2 which lack sequences corresponding to the first Ig domain have been isolated (5, 13–18). Analysis of the genomic structures of FGFR1 and FGFR2 has revealed that this domain is encoded by a single exon that is spliced out in the two Ig domain forms of the receptor (19). The absence of this Ig domain does not appear to affect ligand binding to FGFR1 (13) or FGFR2 (18, 20).

Other splice variants in FGFR1 and FGFR2 arise in Ig domain III (13, 19). The COOH-terminal half of this domain in FGFR1 is encoded by three alternative exons (IIIa, IIIb, and IIIc) that can result in the expression of a secreted receptor and two different transmembrane receptors, respectively. Exons IIIb and IIIc have homologs in the FGFR2 gene (19). Their mutually exclusive splicing in the second half of Ig domain III results in the *bek* (IIIc) isoform (4, 6, 21) or the KGF receptor (KGFR) (IIIb) isoform (15, 17, 18, 22). The *bek* receptor has been shown to bind with high affinity to FGF-1, FGF-2, FGF-4, and FGF-5 but not to FGF-7 (4, 6, 23, 24). In contrast, KGFR binds FGF-7 as well as FGF-1 but the binding to FGF-2 is significantly decreased (18, 25). Recently, the genomic organization of Ig domain III of FGFR3 has revealed the existence of homologous IIIa, IIIb, and IIIc exons (26). The original FGFR3 cDNA containing the IIIc exon is preferentially activated by FGF-1 and FGF-4, to a lesser extent by FGF-2, and has almost no response to FGF-5 (27). The IIIb splice variant of FGFR3, however, shows the most restricted ligand binding properties of any FGFR described so far, binding exclusively FGF-1 (26). The genomic organization of FGFR4 shows the presence of a single exon encoding the COOH-terminal half of the Ig domain III region (28).

A role for FGFs in development is suggested by the unique expression patterns of several family members (29–32). Likewise, the FGFRs have recently been shown to have unique

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¹ The abbreviations used are: FGF, fibroblast growth factor; FGFR, FGF receptor; EGF, epidermal growth factor; PBS, phosphate-buffered saline; KGF, keratinocyte growth factor; KGFR, KGF receptor; CMV, cytomegalovirus; CHO, Chinese hamster ovary; PAGE, polyacrylamide gel electrophoresis.

temporal and spatial expression patterns as well (33–35). In addition, the targeted expression of a dominant negative FGFR1 in the epidermis (36) and FGFR2 in the lung (37) in transgenic mice results in disruptions in the normal developmental architecture of those tissues. FGFs and their receptors have been implicated in amphibian limb regeneration, a process which closely parallels normal limb development. Infusion of FGF into the distal stump of denervated newt limbs stimulates cell cycling over the depressed level normally observed after denervation (38). Recently, FGF-1 (39) and two FGFRs, namely FGFR1 and FGFR2 (22), have been shown to be present in the regenerating limb blastemas of newts, with the latter displaying unique temporal and spatial expression patterns throughout the regeneration process. We further showed that KGFR is the FGFR2 variant that is expressed in the basal layer of the wound epithelium (40). The present study was undertaken to explore the functionality of the newt homolog of KGFR and its potential relevance in amphibian limb regeneration. Here we present data showing that the newt KGFR possesses the ability to bind specific members of the FGF family in a manner indistinguishable from its human counterpart. Moreover, the expression of this receptor in mouse MM14 myoblast cells represses terminal differentiation mediated by FGF-7.

MATERIALS AND METHODS

Plasmid cDNA Construction—The isolation of overlapping, partial cDNAs encoding newt homologs of two FGFR2 splice variants *bek* and KGFR have been described elsewhere (22). These overlapping cDNAs were used to construct the full-length newt KGFR cDNA in pBluescript KS(+) (Stratagene) by conventional DNA cloning techniques (41). The entire nucleotide and amino acid sequences of the newt KGFR have been determined (42). The full-length receptor cDNA construct was excised from pBluescript with *XhoI* and *NotI* and directionally cloned into the *XhoI*-*NotI* sites of the mammalian expression vector pBJ5 (43) to make the newt KGFR expression plasmid pNKGFR. Expression of cDNAs cloned into this vector are driven by the strong SR α hybrid promoter (44). pHKGFR is a human KGFR expression plasmid that contains a three Ig domain form of the human KGFR cDNA (18) cloned into pBJ5.

The *EcoRI* site of a 245-base pair *EcoRI*/*HindIII* fragment of pHCA177CAT (45) containing the region of the human α -cardiac actin gene from -177 base pairs to +68 base pairs was filled in and the insert cloned into the *SmaI*/*HindIII* site at the 5' end of the luciferase gene in the pGL2 basic vector. The resultant plasmid is designated α -cardiac actin/luciferase reporter.

Cell Lines and Transfections—CHO-K1 cells (ATCC, Rockville, MD) were maintained in Ham's F-12 nutrient mixture supplemented with 10% fetal bovine serum and penicillin/streptomycin. CHO cells were cotransfected with 10 μ g of pBJ5 or pNKGFR and 1 μ g of pSV2neo using the modified calcium-phosphate precipitation method (46). Transfected cells were placed under G418 (Life Technologies, Inc.) selection (400 μ g/ml) and colonies isolated 2 weeks later. Stable transfectants were maintained in media supplemented with G418 (400 μ g/ml) and tested for the expression of newt KGFR by binding analysis. A pBJ5 transfectant (CHO/Control) and a newt KGFR expressing cell line (CHO/NKGFR) were selected for further study.

NIH/3T3 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% calf serum and penicillin/streptomycin. A transformed NIH/3T3 cell line, NIH/HKGFR (18), which overexpresses the human KGFR was maintained in the same media with the addition of G418 (750 μ g/ml). Mouse MM14 cells (47) were cultured on gelatin-coated plates in growth medium consisting of Ham's F-10 supplemented with 0.8 mM CaCl₂, 100 units/ml penicillin, 5 μ g/ml streptomycin, and 15% horse serum. The concentration of FGF-2 was increased from 5 to 40 ng/ml with increasing cell density.

Growth Factors—Bovine brain FGF-1 (R & D Systems, Minneapolis MN), human recombinant FGF-2 (48), and human recombinant FGF-7 (Promega, Madison, WI) were iodinated using the chloramine-T method. Briefly, 2 μ g of FGF were incubated together with 10 μ l of 1 M sodium phosphate buffer, pH 7.4, 1 mCi of Na¹²⁵I (Amersham), and 30 μ l of 100 μ g/ml chloramine T in a final volume of 70 μ l for 90 s at room temperature. The reaction was stopped by the addition of 100 μ l of 20 mM dithiothreitol and further incubated at room temperature for 10 min. The iodination reaction was placed over a heparin-agarose column

and washed with wash buffer (20 mM HEPES, 0.2% bovine serum albumin, 0.4 M NaCl) to remove unincorporated ¹²⁵I and biologically inactive ¹²⁵I-FGF. Bound ¹²⁵I-FGF was eluted off the column with elution buffer (20 mM HEPES, 0.2% bovine serum albumin, NaCl; the NaCl concentration varied depending on the ¹²⁵I-FGF that was being eluted off the column: for FGF-1, 2.0 M; FGF-7, 1.0 M; FGF-2, 3.0 M). The biological activity of the labeled FGFs was ascertained by DNA synthesis assays on responsive cell lines. Specific activities ranged from 1 to 5 $\times 10^5$ cpm/ng for ¹²⁵I-FGF-1 and ¹²⁵I-FGF-2 and 4 to 8 $\times 10^4$ cpm/ng for ¹²⁵I-FGF-7. Murine EGF (Promega) was used as a nonspecific competitor in the competition assays.

The FGF-1 protein used in the skeletal muscle reporter gene activity assay was obtained from bovine brain as described elsewhere (48).

Qualitative Binding Assays, Competition Assays, and Scatchard Analysis—NIH/3T3, NIH/HKGFR, CHO/NKGFR, and CHO/Control were seeded at a density of 7 $\times 10^4$ cells/well in a 24-well plate. Twenty-four hours later the cells were washed twice with cold (4 $^{\circ}$ C) phosphate-buffered saline (PBS) and incubated with ¹²⁵I-FGF in 0.2 ml of binding buffer (Ham's F-12 with 25 mM HEPES, pH 7.3, 0.15% gelatin, and 10 μ g/ml heparin) for 2 h at 4 $^{\circ}$ C. Cells were then washed twice with PBS and once with either PBS, 1.0 M NaCl (¹²⁵I-FGF-1); PBS, 0.5 M NaCl (¹²⁵I-FGF-7); or PBS, 2.0 M NaCl (¹²⁵I-FGF-2). Cells were then solubilized in 0.5 ml of 0.3 N NaOH and bound radioactivity counted on a Beckman γ -counter. The addition of a brief salt wash along with the presence of heparin in the binding buffer minimized low affinity binding of the tracers.

For the qualitative binding assays, protein assays were then performed on the solubilized cells as described by the manufacturer (Bio-Rad). Incubation of the cells with the labeled FGFs was carried out in the absence (total binding) or presence (nonspecific binding) of 100-fold molar excess of unlabeled FGF. Mean cpm bound per μ g of total cell protein (\pm S.D.) was determined from triplicate samples. Specific binding is defined as total binding minus nonspecific binding. In the competition assays ¹²⁵I-FGF-1 (2.3 ng/ml) or ¹²⁵I-FGF-7 (3.4 ng/ml) was added to the wells in the presence of increasing amounts of unlabeled competitor. Duplicate wells were used for each concentration of unlabeled competitor. For the Scatchard analysis increasing amounts of ¹²⁵I-FGF were added to duplicate wells with nonspecific binding counts obtained in parallel assays using 100-fold excess of unlabeled FGF.

Cross-linking Analysis—CHO/Control and CHO/NKGFR were seeded in 60-mm dishes at a density of 5 $\times 10^5$ cells/dish. Twenty-four hours later cells were washed once with cold PBS and incubated with 1 $\times 10^6$ cpm of labeled FGFs (in the absence or presence of 100-fold molar excess of unlabeled FGFs) in 5.0 ml of binding buffer for 1 h at 4 $^{\circ}$ C. Cells were washed three times with cold PBS and labeled FGF was cross-linked to cell surface receptors by incubation with 5.0 ml of 0.3 mM disuccinimidyl suberate (diluted in PBS from a 30 mM stock in dimethyl sulfoxide) for 30 min at 4 $^{\circ}$ C. Cells were then washed twice with cold PBS, scraped in 1.5 ml of PBS, and centrifuged for 2 min at 14,000 $\times g$. The cell pellet was resuspended in 45 μ l of lysis buffer (20 mM HEPES, pH 7.4, 150 mM NaCl, 1% Triton X-100, 10% glycerol, 1.5 mM MgCl₂, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 μ g/ml aprotinin, and 1 μ g/ml leupeptin) and incubated on ice for 30 min. Solubilized cells were centrifuged for 5 min at 14,000 $\times g$ and 40 μ l of the clarified cell lysate transferred to 40 μ l of 2 \times SDS sample buffer. Samples were boiled for 5 min and subjected to 7.5% SDS-PAGE after which the gel was dried and exposed to an x-ray film.

Skeletal Muscle Reporter Gene Assay—MM14 cells were seeded at a density of 5 $\times 10^4$ cells per 100-mm dishes 6–8 h before transfection. A calcium phosphate-DNA precipitate containing 1 μ g of α -cardiac actin/luciferase reporter construct (a gift from Dr. Stephen Konieczny, Purdue University) and 0.5 μ g of CMV-LacZ (Centre Commercial de Gros (Toulouse, France)) with 15 μ g of pBJ5, pNKGFR, or pHKGFR was prepared in 0.55 ml of HBS (25 mM HEPES, pH 7.05, 140 mM NaCl, 5 mM KCl, 0.75 mM Na₂HPO₄, 6 mM dextrose) containing 0.11 M CaCl₂. The cells were incubated with 0.5 ml of the precipitate for 20 min before addition of growth medium containing FGF-2. After 4 h, the cells were osmotically shocked for 2.5 min with 15% glycerol in HBS. Growth medium containing 1 μ M insulin and 20 ng/ml FGF-1, FGF-2, or FGF-7 were added as indicated. Additional FGF-1, FGF-2, or FGF-7 was added every 12 h. The cells were harvested for assay of luciferase and β -galactosidase activities 36 h following the osmotic shock. Luciferase activity was measured using the Luciferase Assay System (Promega) following the manufacturer's instructions except for the addition of 2 mM phenylmethylsulfonyl fluoride and 1 μ g/ml leupeptin to the cell solubilization buffer. β -Galactosidase activity was determined in aliquots of the same lysates using the Galacto-Light[®] chemiluminescent reporter assay system (TROPIX). Luciferase and β -galactosidase activ-

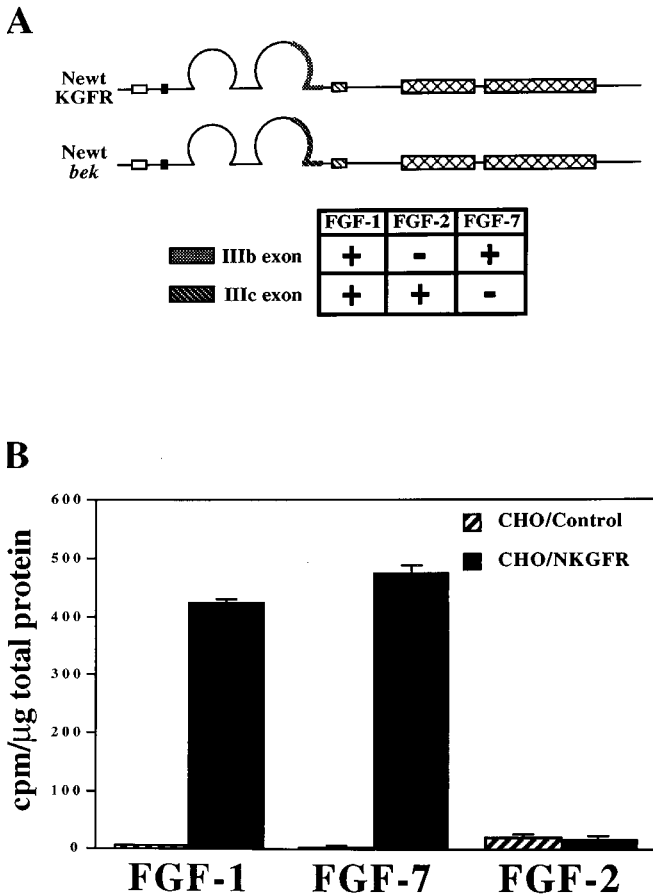


FIG. 1. Newt FGFR2 constructs and qualitative binding assay of newt KGFR expressing cell line. *A*, illustration of the two newt FGFR2 receptor cDNA constructs. For both the *bek* and KGFR constructs the *open boxes* are the signal sequence, the *filled boxes* are the acidic domain, the *hatched boxes* represent the transmembrane domain, and the *cross-hatched boxes* are the kinase domain separated by a kinase insert. The *loops* represent the Ig-like domains. The *line* extending to the *left* of the signal sequence represents an extension of 19 amino acid residues to another in-frame methionine that was included in the cDNAs. The *thicker line* representing the carboxyl-terminal half of the proximal Ig-like domain highlights the area where two mutually exclusive exons (IIIb and IIIc) are spliced into the final transcript. The IIIb exon in newt KGFR is *stippled*, whereas the IIIc exon in newt *bek* is *hatched*. The small table shows the FGF specificity that these two exons impart onto the respective human FGFR2 receptors. *B*, graph showing the ability of the newt KGFR expressing cell line CHO/NKGFR (*filled bar*) to bind to the indicated iodinated FGFs compared to the vector transfected cell line CHO/Control (*hatched bar*).

ities were quantitated in a Berthold Lumat luminometer. Reporter gene activity was calculated by dividing the values obtained for α -cardiac actin/luciferase activity by the CMV/ β -galactosidase activity determined in each extract. The activity of the CMV promoter determined by the β -galactosidase assay in proliferating and differentiated MM14 cells varies less than 2-fold.²

RESULTS

Newt KGFR cDNA Construction—We have previously reported the isolation from the newt, *Notophthalmus viridescens*, of overlapping cDNAs which encode the amphibian homologs of two FGFR2 splice variants, *bek* and KGFR (22). To address the question of whether the potential receptors are functional molecules, the overlapping cDNAs were used to construct the full-length receptors shown in Fig. 1A. The full-length newt KGFR cDNA was originally constructed in pBlue-script and was subsequently cloned into the mammalian

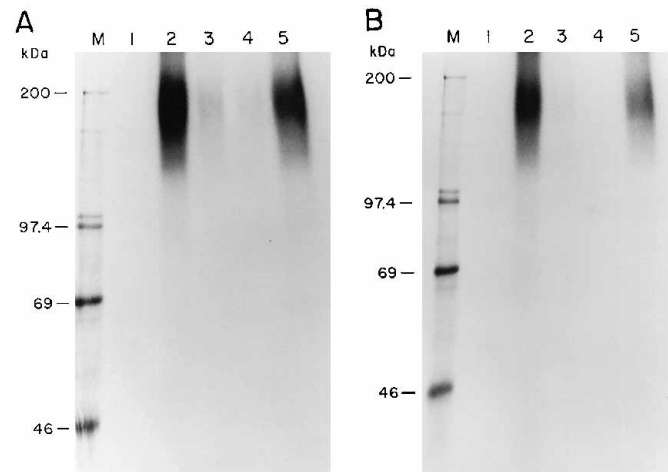


FIG. 2. Cross-linking analysis of the newt KGFR expressing cell line CHO/NKGFR with labeled FGFs. The newt KGFR expressing cell line CHO/NKGFR (*lanes 2-5*) and the vector transfected cell line CHO/Control (*lane 1*) were incubated with ¹²⁵I-FGF-1 (*A*) or ¹²⁵I-FGF-7 (*B*) in the absence (*lanes 1 and 2*) or presence of a 100-fold molar excess of unlabeled competitor: FGF-7 (*lane 3*), FGF-1 (*lane 4*), and FGF-2 (*lane 5*). The cells were incubated with the FGFs for 1 h at 4 °C after which they were cross-linked to cell surface receptors with DSS (0.3 mM). The cross-linked products were then analyzed by 7.5% SDS-PAGE. The molecular masses of the protein standards (*lane M*) are in kDa.

expression vector pBJ5. Cotransfection of CHO-K1 cells with pSV2neo and either the pBJ5 expression vector or the newt KGFR expression construct, pNKGFR, resulted in the isolation of stable G418-resistant transfectants CHO/Control and CHO/NKGFR, respectively.

Binding Properties of the Newt KGFR—To evaluate the ability of the newt KGFR to bind to FGF-1 and FGF-7, a series of binding assays were performed on the CHO/Control and CHO/NKGFR cell lines using ¹²⁵I-FGFs as tracers. As a comparison, a cell line overexpressing the human KGFR, NIH/HKGR, and its parental NIH/3T3 cell line were used in these assays. The NIH/HKGR cell line has been previously shown to bind to FGF-7 with high affinity (18).

In a qualitative binding assay the human KGFR showed a significant increase in binding to ¹²⁵I-FGF-1 and ¹²⁵I-FGF-7 relative to NIH/3T3 cells but was not capable of binding ¹²⁵I-FGF-2 over that which was observed in the parental cell line (data not shown). These results confirmed the previously published results of this human FGFR2 splice variant (18). Likewise, the newt KGFR expressing cell line, CHO/NKGFR, shows an increase in binding to ¹²⁵I-FGF-1 and ¹²⁵I-FGF-7 but not ¹²⁵I-FGF-2 when compared to the expression vector transfected cell line, CHO/Control (Fig. 1B). Thus, the newt KGFR exhibits an FGF binding profile that is similar to its human counterpart.

To determine the size of the newt KGFR expressed in the CHO/NKGFR cell line, a cross-linking analysis was performed. The predicted size of the newt KGFR based on the primary amino acid sequence of the cDNA is 82 kDa. When either ¹²⁵I-FGF-1 or ¹²⁵I-FGF-7 was cross-linked to the newt KGFR and analyzed by 7.5% SDS-PAGE, a predominant cross-linked product with an apparent molecular mass of 165–170 kDa was detected (*lanes 2*, Fig. 2, *A* and *B*, respectively). Subtraction of the molecular mass of the ligands yields an estimated molecular mass of 150 kDa for the expressed receptor. The difference between the apparent and predicted molecular weights for newt KGFR is most likely due to an extensive degree of *N*-linked glycosylation in the extracellular domain. A cross-linked product is not observed in the CHO/Control cell line with either

² K. Hannon, A. J. Kudla, M. J. McAvoy, K. L. Clase, and B. B. Olwin, manuscript submitted.

of the labeled FGFs (*lanes 1, Fig. 2, A and B*). Cross-linkings carried out in the presence of 100-fold excess of unlabeled FGFs indicate that the receptor is specific for FGF-1 and FGF-7 as both of these ligands are capable of competing with either ^{125}I -FGF-1 or ^{125}I -FGF-7 for the receptor (*lanes 3 and 4 of Fig. 2, A and B*). Interestingly, FGF-1 appears to be a more effective competitor than FGF-7 for ^{125}I -FGF-7 which may reflect a higher affinity of the receptor for FGF-1. Unlabeled FGF-2 is a much less effective competitor for both ^{125}I -FGF-1 and ^{125}I -FGF-7 binding to the newt KGFR (*lane 5, Fig. 2, A and B*).

To address the specificity of the newt KGFR in a quantitative manner, a competition assay was performed on CHO/NKGFR cells using ^{125}I -FGF-1 and ^{125}I -FGF-7 as radiolabeled tracers in the presence of increasing concentrations of unlabeled FGF-1, FGF-2, FGF-7, or EGF. High affinity ^{125}I -FGF-1 binding to CHO/NKGFR cells was effectively competed by FGF-1 and FGF-7 with similar efficiencies (50% displacement at 35 ng/ml for FGF-1 and 60 ng/ml for FGF-7). However, neither FGF-2 nor EGF was able to compete for ^{125}I -FGF-1 binding to newt KGFR on these cells (*Fig. 3B*). In a similar manner, high affinity binding of ^{125}I -FGF-1 to NIH/HKGFR cells was also competed by FGF-1 and FGF-7 (*Fig. 3A*). Although EGF again showed no specific competition toward human KGFR, FGF-2 was capable of competing with ^{125}I -FGF-1 at higher concentrations. When ^{125}I -FGF-7 is used as the tracer a dramatic difference in the pattern of competition can be seen with FGF-1 and FGF-7 in that FGF-1 appears to be a more effective competitor than FGF-7 for binding of ^{125}I -FGF-7 to both human KGFR (*Fig. 4A*) and newt KGFR (*Fig. 4B*). This data is consistent with the observations from the cross-linking analysis. The concentrations of unlabeled FGF-1 and FGF-7 needed to achieve 50% displacement of the two labeled tracers were consistently 3–4-fold higher for the newt KGFR.

The results of the competition assay suggest that the newt KGFR has a slightly lower affinity toward FGF-1 and FGF-7 relative to its human counterpart. To more precisely determine the affinity of FGF-1 and FGF-7 to the newt KGFR a Scatchard analysis was performed on the CHO/NKGFR cell line. *Fig. 5, A and B*, reveal that the newt KGFR binds to FGF-1 with a K_d of 660 pM and to FGF-7 with a K_d of 860 pM. The affinity of the newt KGFR for FGF-7 is approximately 4-fold lower than the reported affinity of the human KGFR for this ligand (18).

Ability of Newt KGFR to Repress Differentiation in MM14 Mouse Myoblasts—The above data indicate that the newt KGFR binds FGF family members with high affinity in a manner similar to the human KGFR. To address whether this binding results in activation of the receptor and subsequent intracellular signaling, we tested the human and newt KGFR for their ability to repress differentiation in the mouse MM14 myoblast cell line. This cell line is dependent on FGFs for repression of skeletal muscle differentiation. Deprivation of FGF induces terminal differentiation that results in the exit of the cells from the cell cycle, expression of muscle-specific genes, and fusion into multinucleated myotubes (49). The FGFs capable of repressing differentiation in MM14 cells include FGF-1, FGF-2, and FGF-4; however, FGF-7 is not active on these cells (50). Therefore, expression of KGFR by MM14 cells should allow us to assay its ability to repress differentiation in the presence of FGF-7. To assay for differentiation, a luciferase reporter construct was utilized. This construct has a 245-base pair fragment corresponding to the region of the human α -cardiac actin gene from -177 to +68 inserted at the 5' end of the luciferase gene in the pGL2basic luciferase vector. The region -177 to +68 of the human α -cardiac actin gene is sufficient to drive differentiated muscle-specific expression of chloramphenicol acetyltransferase in a mouse myoblast cell line (45). There-

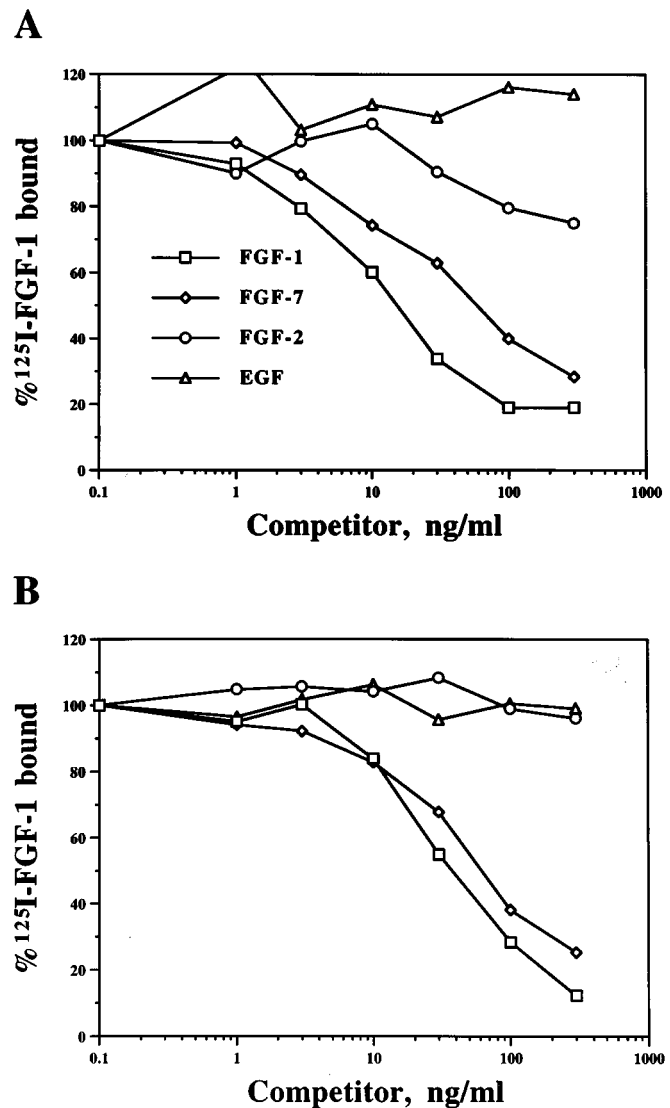


FIG. 3. Competition assay comparing human and newt KGFRs with ^{125}I -FGF-1 as the tracer. The human KGFR expressing cell line NIH/HKGFR (*A*) and newt KGFR expressing cell line CHO/NKGFR (*B*) were incubated with a constant amount of ^{125}I -FGF-1 (2.3 ng/ml) in the presence of increasing concentrations of unlabeled competitors: FGF-1 (open squares), FGF-7 (open diamonds), FGF-2 (open circles), and EGF (open triangles). After washing the cells to remove unbound ligands and solubilizing in 0.3 N NaOH, the samples were counted on a Beckman γ -counter. Each concentration of competitor was carried out in duplicate and the average plotted as a percentage of bound ^{125}I -FGF-1 in the presence of the lowest concentration of competitor. The standard error of the mean for all samples never exceeded 11% of the average.

fore, MM14 cells transfected with an α -cardiac actin/luciferase reporter construct should exhibit an increase in luciferase activity if allowed to differentiate. However, if they are cultured in the presence of the appropriate FGF little increase in luciferase activity will be seen indicating that differentiation is repressed.

MM14 cells transfected with the pBJ5 expression vector along with the α -cardiac actin/luciferase reporter construct exhibit a large increase in luciferase activity, both in the absence and presence of FGF-7 (*Fig. 6*). These results verify that these cells do not express endogenous receptors for FGF-7 and are not capable of responding to this growth factor. As expected, when cultured in the presence of either FGF-1 or FGF-2, the pBJ5 transfected cells are repressed from differentiation as indicated by a low level of luciferase activity. The ability of both FGF-1 and FGF-2 to repress differentiation in

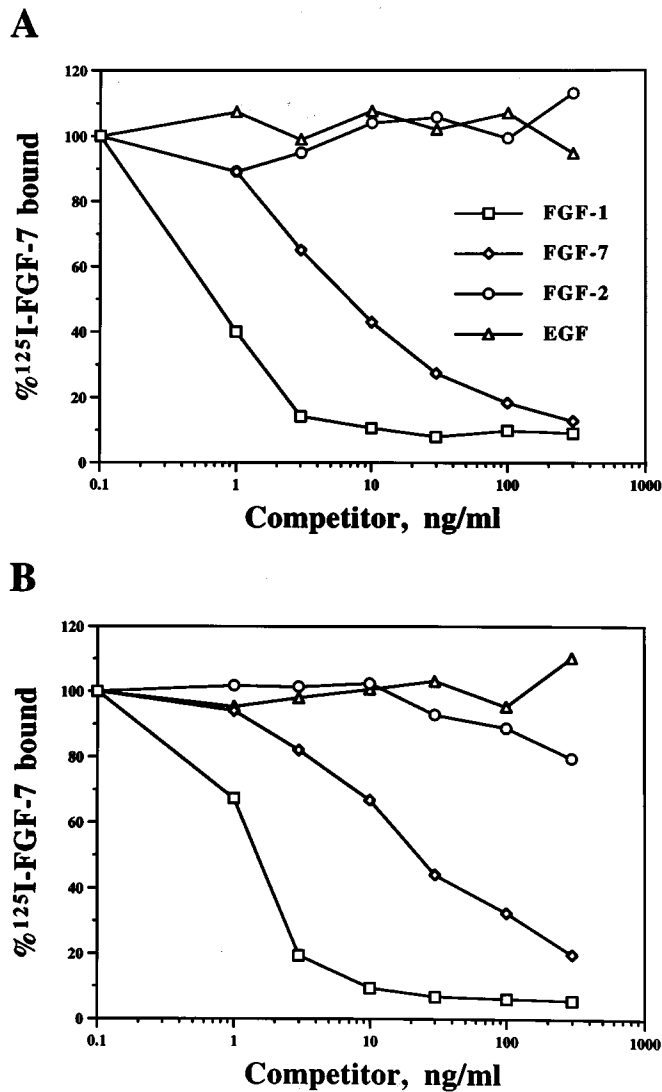


FIG. 4. Competition assay comparing human and newt KGFRs with ^{125}I -FGF-7 as the tracer. The human KGFR expressing cell line NIH/HKGR (A) and newt KGFR expressing cell line CHO/NKGR (B) were incubated with a constant amount of ^{125}I -FGF-7 (3.4 ng/ml) in the presence of increasing concentrations of unlabeled competitors: FGF-1 (open squares), FGF-7 (open diamonds), FGF-2 (open circles), and EGF (open triangles). After washing the cells to remove unbound ligands and solubilizing in 0.3 N NaOH, the samples were counted on a Beckman γ -counter. Each concentration of competitor was carried out in duplicate and the average plotted as a percentage of bound ^{125}I -FGF-7 in the presence of the lowest concentration of competitor. The standard error of the mean for all samples never exceeded 10% of the average.

this assay serves as an internal positive control for the competence of these cells to respond to exogenously added FGF. The expression of either human or newt KGFR by MM14 cells resulted in low levels of luciferase activity when cultured in the presence of FGF-7. The data are indicative of an acquired ability of these transfected cells to repress differentiation mediated by FGF-7 (Fig. 6). In the absence of exogenously added growth factor, both the human and newt KGFR transfected cells showed an increase in luciferase activity. These data suggest that the human as well as the newt KGFR isoform of FGFR2 is capable of mediating a signal within MM14 myoblasts that leads to repression of differentiation. The ability of the ectopically expressed KGFRs to function in this capacity is similar to or better than the activity observed with the endogenous FGF receptors expressed by this cell line.

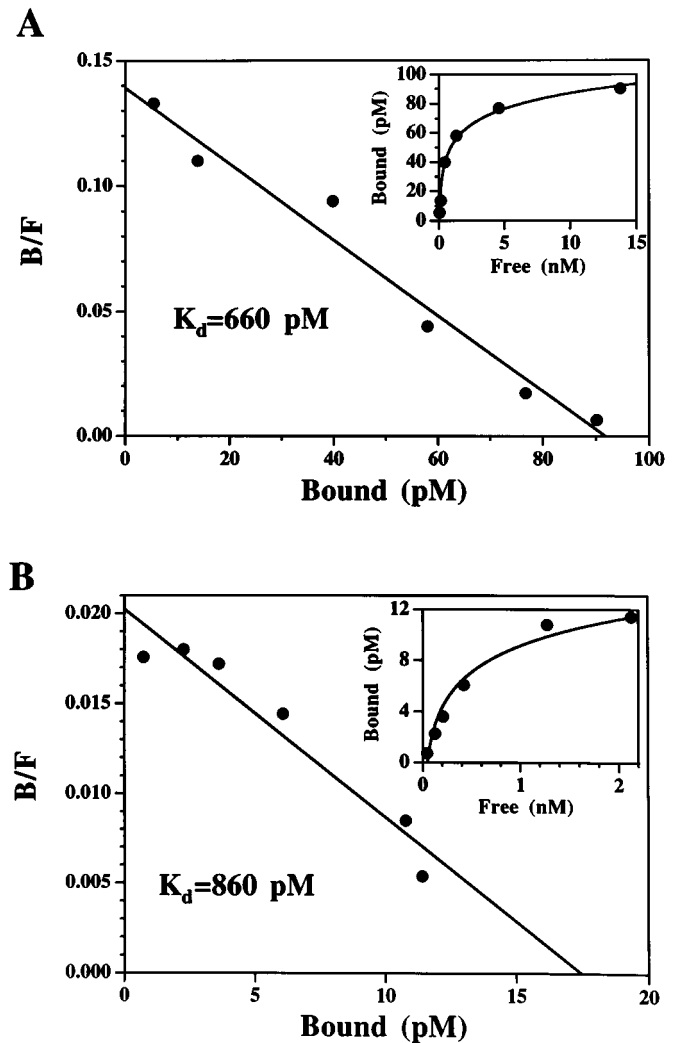


FIG. 5. Affinity of newt KGFR to mammalian FGFs. Newt KGFR expressing CHO/NKGR cells were incubated with increasing concentrations of ^{125}I -FGF-1 (A) and ^{125}I -FGF-7 (B) in the absence (total binding) or presence (nonspecific binding) of a 100-fold excess of unlabeled self. Specific binding is defined as total binding minus nonspecific binding. Duplicate wells were used for each concentration of labeled FGF. Saturation isotherms for both labeled FGFs are shown as insets to the corresponding Scatchard plots. The dissociation constants for both FGF-1 and FGF-7 are indicated in the appropriate graphs. The standard error of the mean for samples of both total and nonspecific binding never exceeded 8% of the average. Receptor numbers per cell obtained with FGF-1 and FGF-7 were 112,000 and 13,000, respectively.

DISCUSSION

In this study, we report on the functional characterization of a newt FGFR2 variant which was previously isolated in our laboratory (22). The construction of full-length cDNAs from the available overlapping cDNA clones resulted in receptors that lacked the first Ig domain and as a consequence the extracellular region of these receptors contain the acidic box followed by Ig domains II and III (Fig. 1A). The receptors differ only in the COOH-terminal half of Ig domain III where it is known that the alternative splicing of two different exons, IIIb and IIIc, takes place in a mutually exclusive fashion giving rise to the KGFR and *bek* variants, respectively. The newt KGFR possesses a unique spatial and temporal pattern of expression in the regenerating newt limb with the strongest expression observed predominantly in the basal layer of the wound epithelium (40). Because the wound epithelium is a necessary component of the regenerate (51) and has been shown to ex-

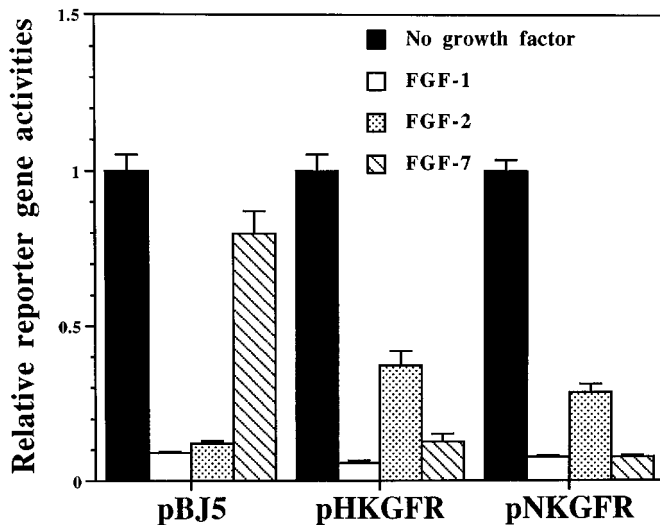


FIG. 6. FGF-7 mediated repression of skeletal muscle differentiation in MM14 cells expressing newt and human KGFR. MM14 myoblasts were transfected with an α -cardiac actin/luciferase reporter plasmid, a CMV-LacZ plasmid, and the pBJ5 expression vector, the newt KGFR expression plasmid pNKGFR, or the human KGFR expression plasmid pHKGFR as described under "Materials and Methods." The transfected cells were cultured either in the absence of any exogenously added growth factor (filled bars) or in the presence of FGF-1 (open bars), FGF-2 (stippled bars), or FGF-7 (hatched bars). Cells were then harvested and assayed for luciferase and β -galactosidase activities. Reporter gene activity represents α -cardiac actin/luciferase activity of a given cell extract divided by the CMV/ β -galactosidase activity measured for that extract. The value obtained for each set of transfected cells cultured in the absence of growth factors was set at 1.0. The actual values of the pNKGFR and pHKGFR transfectants cultured with no growth factor were 72 and 62% that of the pBJ5 transfectant, respectively. We showed that proliferating MM14 cells express FGF-7.³ The FGF-7 that the MM14 cells are expressing may be sufficient to activate the transfected KGFRs and partially repress activation of the reporter construct. The results shown are representative of three individual experiments.

press a number of molecules which are not expressed in skin epidermis (52–54), we were interested in examining the functional capacity of the newt KGFR splice variant. Because no amphibian FGFs are available, mammalian FGFs were utilized as radiolabeled ligands in binding and functional assays. We were encouraged with this approach by the fact that radiolabeled bovine FGF-1 had been used successfully as a probe in *in situ* studies with axolotl limb blastemas (39) and because of the high degree of amino acid identity between human and newt KGFR (22).

In qualitative binding assays using iodinated FGFs we found that a newt KGFR expressing CHO cell line, CHO/NKGFR, exhibited an increase in binding to FGF-1 and FGF-7 relative to a control cell line, CHO/Control (Fig. 1B). However, CHO/NKGFR showed no appreciable increase in binding to FGF-2. These results were indistinguishable from the binding of these FGFs to a human KGFR expressing cell line NIH/HKGFR (data not shown). These results combined with the high degree of amino acid sequence identity observed between the human and newt exon IIIb region (86%), strongly support our claim that this newt FGFR2 splice variant is a functional homolog of the human KGFR.

Cross-linking of either ¹²⁵I-FGF-1 or ¹²⁵I-FGF-7 to receptors on the surface of CHO/NKGFR show a predominant cross-linked product with an apparent molecular mass of 150 kDa. This cross-linked product was specific since the addition of a 100-fold molar excess of unlabeled FGFs prior to cross-linking competed the radiolabeled ligand. Unlabeled FGF-1 and FGF-7 were able to decrease binding of the two labeled FGFs; how-

ever, FGF-2 was not as effective at competing for ¹²⁵I-FGF-1 or ¹²⁵I-FGF-7. These results support the qualitative binding assay that the newt KGFR is specific for FGF-1 and FGF-7. Interestingly, in the presence of ¹²⁵I-FGF-7, FGF-1 appears to be a more effective competitor than FGF-7 for binding to the newt KGFR. This could reflect the fact that the newt KGFR may have a higher affinity for FGF-1 than FGF-7. Alternatively, this could be due to differences in newt and mammalian FGFs. The size of the 150-kDa band is greater than expected for a two Ig domain form of the newt KGFR based on its primary amino acid sequence. Likewise, other investigators have shown the two Ig domain form of FGFR2 cross-linked to labeled FGFs migrating with an apparent size that is larger than predicted from their primary amino acid sequences (17, 20). This difference in size is likely to be due to *N*-linked glycosylation of the receptors as they have a number of potential *N*-linked glycosylation sites in their extracellular domain. The newt KGFR has nine potential *N*-linked glycosylation sites, whereas the two Ig domain form of human *bek* and KGFR have six and seven, respectively. The cDNA encoding the newt KGFR that was cloned into the pBJ5 expression vector does harbor an upstream in-frame methionine which could code for an additional 19 amino acids (42) if translation were to start here. However, the amino acids that follow this methionine do not exhibit an overall hydrophobic character and would not be expected to function as a secretory signal sequence. Interestingly, the position of this upstream methionine is conserved in FGFR2 cDNAs isolated from other species, which may indicate that it has some biological importance. At any rate, the higher molecular weight exhibited by the newt KGFR suggests more extensive post-translational modification. However, this modification does not affect either the biochemical properties or functionality of this receptor (see below).

The specificity of the newt KGFR to different FGFs was demonstrated in a more quantitative manner by competition assays using labeled FGF-1 and FGF-7 as tracers. Unlabeled FGF-1 and FGF-7 were effective in competing with the two labeled FGFs for binding to the newt KGFR, whereas FGF-2 was not an effective competitor at the concentrations used. The human KGFR exhibited the same specificity for binding to FGF-1 and FGF-7 and confirms the results obtained by others (18). As previously observed in the cross-linking analysis, FGF-1 appears to be a more effective competitor than FGF-7 for binding of the newt KGFR to labeled FGF-7. The apparent higher affinity of the newt KGFR toward FGF-1 was confirmed by Scatchard analysis as FGF-1 and FGF-7 exhibited dissociation constants of 660 and 860 pM, respectively.

To address whether the newt KGFR was capable of transducing a signal to the interior of a cell after binding to its ligand, a functional assay was carried out in mouse MM14 myoblast cells transiently transfected with the newt KGFR expression vector and an α -cardiac actin/luciferase reporter plasmid. In the presence of exogenously added FGF-7, both the newt KGFR and human KGFR were capable of transducing the signal required for repressing differentiation in these cells. This was somewhat intriguing because the KGFR isoform of FGFR2 is almost exclusively expressed in cells of epithelial origin. In fact, FGFR1 is likely to be the only FGFR expressed in MM14 cells (55).³ Thus, despite the difference between the cytoplasmic portions of KGFR and FGFR1, these two receptors converge to the same signaling pathway within MM14 cells.

In conclusion, we provide evidence that a newt KGFR previously isolated in our laboratory is a functional receptor in that it is capable of binding FGF-1 and FGF-7 with high affinity but

³ A. J. Kudla and B. B. Olwin, unpublished results.

not FGF-2 and is competent in transducing a proliferative signal in cells expressing it. These results when combined with the unique expression pattern observed in regenerating amphibian limbs indicate that KGFR may have a significant role within the wound epithelium early in the regeneration process. The appearance of the KGFR splice variant of FGFR2 in the basal layer of the wound epithelium during amphibian limb regeneration (40) is reminiscent of the expression of FGFR2 in the epidermis in a wound healing study (56). In this study FGF-7 was shown to be induced 160-fold within the dermis of the skin injury, whereas FGF-1, FGF-2, and FGF-5 were induced only 2–10-fold, and FGF-3, FGF-4, and FGF-6 were undetected in normal and wounded skin. This suggests a possible paracrine role for the dermally expressed FGF-7 acting on the overlying epidermis during wound healing. In a similar fashion, an amphibian FGF-7 or FGF-7-like molecule may be involved in the regenerating amphibian limb. We hypothesize that this putative amphibian FGF-7 may act during the early stages of regeneration in establishing and/or maintaining the wound epithelium which is expressing KGFR.

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