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Heterogeneity in the expression of fibroblast growth factor receptors during limb regeneration in newts (*Notophthalmus viridescens*)

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SUMMARY

Two closely related fibroblast growth factor receptors, FGFR1 and FGFR2, have been cloned from a newt (*Notophthalmus viridescens*) limb blastema cDNA library. Sequence analysis revealed that we have isolated both the *bek* and KGFR variants of FGFR2. These two variants differ only in the second half of the last of their three Ig-like domains. The expression patterns of FGFR1 and FGFR2 during limb regeneration have been determined by in situ hybridization. During the pre-blastema stages of regeneration, FGFR2 expression is observed in the basal layer of the wound epithelium and in the cells of the periosteum. As regeneration progresses to the blastema stages, FGFR2 expression continues to be observed in the basal layer of the wound epithelium with additional hybridization seen in the blastema mesenchyme closely associated with the bisected bones. From the early bud to the mid-bud blastema stage, FGFR1 expression is observed throughout the blastema mesenchyme but, unlike FGFR2, is distinctly absent from the wound epithelium. In the differentiation stages of regeneration, the mesenchymal expression of FGFR2 becomes restricted to the cells of the condensing cartilage and later to the perichondrium. During these later stages of regeneration, the wound epithelium hybridization to the FGFR2 probe is no longer observed. The expression patterns of these receptors suggest that FGFR1 and FGFR2 have distinct roles in limb regeneration, despite their sharing a number of the FGF ligands. Further investigation regarding the potential sources of the FGF ligands will help establish the role that FGFs and FGFRs play in limb regeneration.

Key words: FGFR1, FGFR2, KGFR, limb regeneration, blastema, in situ hybridization

INTRODUCTION

The fibroblast growth factor (FGF) family contains eight members (Basilico and Moscatelli, 1992). The two prototypic members, acidic FGF (Jaye et al., 1986; Wang et al., 1989) and basic FGF (Abraham et al., 1986) have no signal peptide but the remaining six members, FGF-3 (Dickson and Peters, 1987; Acland et al., 1990), FGF-4 (Delli Bovi et al., 1987; Taira et al., 1987), FGF-5 (Zhan et al., 1988), FGF-6 (de Lapeyriere et al., 1990), keratinocyte growth factor (KGF) (Finch et al., 1989) and androgen-induced growth factor (AIGF) (Tanaka et al., 1992) all have signal peptides. Various members of the FGF family are involved in cell growth, differentiation and survival as well as embryonic induction and angiogenesis (reviewed in Basilico and Moscatelli, 1992). Because the release of aFGF and bFGF is thought to be through dead or dying cells, it is implied that they are also involved in tissue repair. Moreover, KGF mRNA has been shown to be induced more than 160-fold during wound healing (Werner et al., 1992).

As with most polypeptide growth factors, the FGF signal is transduced via membrane-spanning protein tyrosine kinase (PTK) receptors (reviewed in Johnson and Williams, 1993). The four members of the FGF receptor (FGFR) family, *flg*/FGFR1 (Ruta et al., 1989; Dionne et al., 1990; Johnson et al., 1990), *bek*/FGFR2 (Kornbluth et al., 1988; Dionne et al. 1990), FGFR3 (Keegan et al., 1991) and FGFR4 (Partanen et al., 1991), all contain three immunoglobulin (Ig)-like extracellular domains (Williams and Barclay, 1988). The first Ig-like domain may or may not be present due to alternative splicing, resulting in either a two or three loop variant (Mansukhani et al., 1990; Fujita et al., 1991). This first loop has no effect on ligand binding and its function remains unknown (Johnson et al., 1990; Mansukhani et al., 1990). The genes of FGFR1 and FGFR2 contain three consecutive yet mutually exclusive exons that encode the 3′ half of the last Ig-like domain (Champion-Arnaud et al., 1991; Eisemann et al., 1991; Johnson et al., 1991; Yayon et al., 1992). Alternative splicing in this region generates secreted forms of these receptors and receptors with differences in their FGF-binding specificities. Splicing of the first of the three exons (IIIa) into the mRNA results in a secreted form of the receptor containing no transmembrane or PTK domain (Johnson et al., 1990, 1991). If the

next exon (IIIb) is spliced into the mRNA, a membrane spanning PTK receptor with a high affinity for aFGF and KGF results. When considering FGFR2, this isoform is referred to as the KGF receptor (Miki et al., 1991, 1992; Yayon et al., 1992). Inclusion of the last of these three exons (IIIc) confers high affinity to aFGF, bFGF and FGF-4 (Dionne et al., 1990; Mansukhani et al., 1992) but not to KGF (Miki et al., 1992). This FGFR2 isoform is referred to as a '*bek*-like' receptor.

Expression patterns of several FGF proteins during development are well documented (reviewed in Whitman and Melton, 1989; Hebert et al., 1990; Niswander and Martin, 1992; Tannahill et al., 1992;). The FGFs have also been implicated in amphibian limb regeneration but their specific role in this developmental process remains obscure. When FGF is infused into the distal stump of denervated newt limbs, cell cycling is stimulated over the depressed level normally seen after denervation (Mescher and Gospodarowicz, 1979; Gospodarowicz and Mescher, 1980). By binding assays and western blotting analysis, Boilly et al. (1991) showed that aFGF and its receptor(s) are present within the newt limb blastema, nevertheless, the cellular source of this growth factor was not determined. In the mouse limb bud, FGFR2 transcripts were detected in the surface ectoderm, whereas FGFR1 transcripts were distributed diffusely in the mesenchyme (Orr-Urtreger et al., 1991; Peters et al., 1992). The goal of the present study was to determine whether the FGFR expression patterns of developing limbs would be reprogrammed during limb regeneration. We have cloned cDNAs of newt FGFR1 and FGFR2 and utilized riboprobes made from these cDNAs to carry out in situ hybridization at various stages of newt limb regeneration. The results suggest multiple roles for the FGFs and their receptors during the limb regeneration process.

MATERIALS AND METHODS

Animals

Adult newts, *Notophthalmus viridescens*, were collected in southern Ohio, maintained in aged tap water at room temperature and fed raw beef liver four times a week. Limbs were amputated through the midradius/ulna and protruding bones were trimmed to the level of soft tissues. Newts were then returned to water and allowed to regenerate to the desired stage. Regenerates were collected at 5 and 10 days after amputation (preblastema stages), and at early-bud, mid-bud, late-bud, palette and digit stages (staged according to Iten and Bryant, 1973). From two to six limbs/regenerates were sampled at each stage. Operations were performed while animals were anesthetized with MS-222 (ethyl *m*-aminobenzoate methanesulfonate; Sigma, St Louis, MO).

Cloning and sequencing of newt FGFR1 and FGFR2

A 2.0 kbp *Eco*RI fragment from a human FGFR1/*flg* cDNA clone (Ruta et al., 1988) was used to screen a newt mid-bud blastema cDNA library constructed in λgt11 (Ragsdale et al., 1989). A total of 5×10^5 plaques (5×10^4 plaques/150 mm plate) were transferred to duplicate nitrocellulose filters (Schleicher & Schuell) and hybridized to a random primed human FGFR1 cDNA probe under low stringency (43% formamide, 5× SSC, 5× Denhardt's solution, 1% SDS, 200 μg/ml salmon sperm DNA in 50 mM phosphate buffer, pH 6.5 at 37°C). Hybridized filters were washed for 1 hour at 37°C in 2× SSC/0.1% SDS and exposed to Kodak X-OMAT AR film overnight. The *Eco*RI phage inserts from isolates 102, 108 and 109 were subcloned into the *Eco*RI site of pBluescript KS(+) (Stratagene) or subcloned directly into the *Eco*RI site of pBR322 derived from the *E. coli* strain Y1088 (Chiu et al., 1992) for DNA sequence analysis. One of the two *Eco*RI sites of phage clone 110 was missing and the insert cDNA could not be excised by *Eco*RI digestion. Therefore, primers flanking the phage *Eco*RI cloning site were used in polymerase chain reactions (PCR) to amplify the phage insert. A single band was isolated and cloned into the *Hin*dII site of pBluescript KS(+). Rescreening of the cDNA library was carried out under high stringency using the 240 bp *Eco*RI-*Bam*HI fragment of Clone 109 as a probe. Nested deletions of the phage clones 102, 109, 110 and 310 were generated in both orientations using the method of Henikoff (1984). Double and single-stranded DNA from selected clones were sequenced using the dideoxy method (Sanger et al., 1977) and Sequenase (USB).

RNA isolation and northern hybridization

RNA was isolated from frozen tissues by acid guanidinium isothiocyanate extraction followed by CsCl gradient centrifugation (Chirgwin et al., 1979). RNA samples were electrophoresed through 1.0% agarose/formamide gels, transferred to Hybond-N nylon membranes and probed with antisense [α-^{32}P]UTP-labeled riboprobes. The filters were washed twice in 2× SSC/0.1% SDS for 15 minutes at 65°C and twice in 0.1× SSC/0.1% SDS for 15 minutes at 65°C. The membranes were then exposed to Kodak X-OMAT AR film and developed.

In situ hybridizations

To generate FGFR2-specific riboprobes, a 306 bp *Bbs*I-*Bam*HI fragment from Clone 310 was subcloned into the *Sma*I site of pBluescript SK(+) and designated MP70-1. To generate an antisense transcript, 1 μg of MP70-1 was digested with *Eco*RI and in vitro transcription was carried out using T3 RNA polymerase according to the manufacturer's protocol (Stratagene). The sense strand transcript was generated by digestion of MP70-1 with *Bam*HI followed by in vitro transcription using T7 RNA polymerase. FGFR1-specific riboprobes were generated using the exonuclease III/mung bean nuclease generated deletion clones used in the sequencing reactions (see above) that allowed the synthesis of the sense and antisense transcripts representing the 73 amino acid carboxyl terminal tail of the receptor and 80 nucleotides of the 3′-untranslated sequence. All transcription reactions were performed in the presence of [^{35}S]UTPαS. The RNA probes were purified by Nuctrap push columns (Stratagene).

Blastemas with a small amount of stump tissue were isolated and fixed in 4% paraformaldehyde containing 1× PBS, pH 7.2, for 2 hours at 4°C. The blastemas were then washed two times in 1× PBS at 4°C for 30 minutes, and frozen on a dry ice/isopropanol slurry in OCT compound. 10 μm cryosections were placed on TESPA (3-triethoxysilylpropylamine)-treated slides and fixed for 20 minutes in 4% paraformaldehyde containing 1× PBS. The slides were dehydrated through graded ethanol and stored at −80°C until hybridization. Prior to hybridization the sections were treated with proteinase K (20 μg/ml) for 10 minutes at 37°C, acetylated by immersing slides in 0.25% acetic anhydride in 0.1 M triethanolamine buffer, pH 8.0 for 20 minutes and dehydrated through graded ethanol solutions. The hybridizations were carried out at 50-55°C in hybridization mix (50% formamide, 0.3 M NaCl, 10 mM Tris-HCl, 1× Denhardt's solution, 5 mM EDTA, 0.5 mg/ml yeast tRNA, 10% dextran sulfate, pH 7.5) with either sense or antisense riboprobes at a concentration of 1×10^7 cts/minute/ml for 16 hours.

Slides were washed in 4× SSC for 5 minutes at room temperature before a 30 minutes wash in 50% formamide/2× SSC/0.1% 2-mercaptoethanol at 50-55°C. The slides were then treated with RNase A (20 μg/ml) for 30 minutes at 37°C. The slides were

further washed in 50% formamide/2× SSC/0.1% 2-mercaptoethanol for 30 minutes at 50-55°C, 2× and 0.1× SSC each for 15 minutes at 50-55°C. The sections were then dehydrated, coated with Kodak NTB-2 emulsion diluted 1:1 with distilled water and exposed for 15 to 20 days. Slides were developed with Kodak D-19 developer for 2.5 minutes at 15°C and fixed for 5 minutes with Kodak fixer. Sections were stained with hematoxylin and counterstained with eosin. The sections were visualized with both dark- and light-field microscopy.

RESULTS

Cloning and characterization of NvFGFR1 and NvFGFR2

A newt forelimb mid-bud blastema cDNA library was screened under reduced stringency with a partial human cDNA fragment of the FGFR1 gene. Four lambda clones, 102, 108, 109 and 110, were purified and sequenced. The sequences show that three clones, 108, 109 and 110, overlap each other and are most homologous to the FGFR2 cDNAs of different species (Fig. 1A). The sequence of clone 102 (accession no. L19868) indicates that this cDNA is most similar to FGFR1 cDNAs of different species. These clones all contain the cytoplasmic tyrosine kinase domain but are truncated in the extracellular ligand-binding domain. Two of the newt FGFR2 clones, 109 and 110, also contain a region in the 3′ half of the distal Ig-like domain that vary between them. These variants represent the newt cognates of two different isoforms of FGFR2, one homologous to *bek* the other to the KGFR (Fig. 1). To obtain full-length FGFR2 cDNA clones, the 5′ portion of clone 109 (240 bp *Eco*RI-*Bam*HI) was used to rescreen the blastema cDNA library and three more cDNA clones were isolated, purified and sequenced. Clones 302 and 310 represent the two Ig-like loop variant of FGFR2 while clone 301 represents a truncated form of the three loop form of FGFR2. All three 300 series cDNA clones are of the KGFR isoform (Fig. 1A).

The DNA sequences for both the *bek* (accession no. L19869) and KGFR (accession no. L19870) of newt FGFR2 containing two Ig-like loops have been submitted to GenBank. Fig. 1B shows the difference between the newt KGFR-like and *bek*-like forms of FGFR2. The amino acid alignment, as well as the nucleotide sequence comparisons, show that the flanking regions of these cDNAs are identical whereas the region in between shares 58% amino acid similarity (Fig. 1B). It is noted that the sequence similarity is greater, 73% and 78%, respectively (Fig. 1C,D), between the same isoforms of different species (newt KGFR versus human KGFR and newt *bek* versus human *bek*) than between the different isoforms of the same species (Fig. 1B).

Comparison of amino acid identity of various regions of KGFR variants of newt FGFR2 with the four known human FGF receptors clearly indicates that we have isolated the newt FGFR2 cDNA (Fig. 2). As such, the overall amino acid

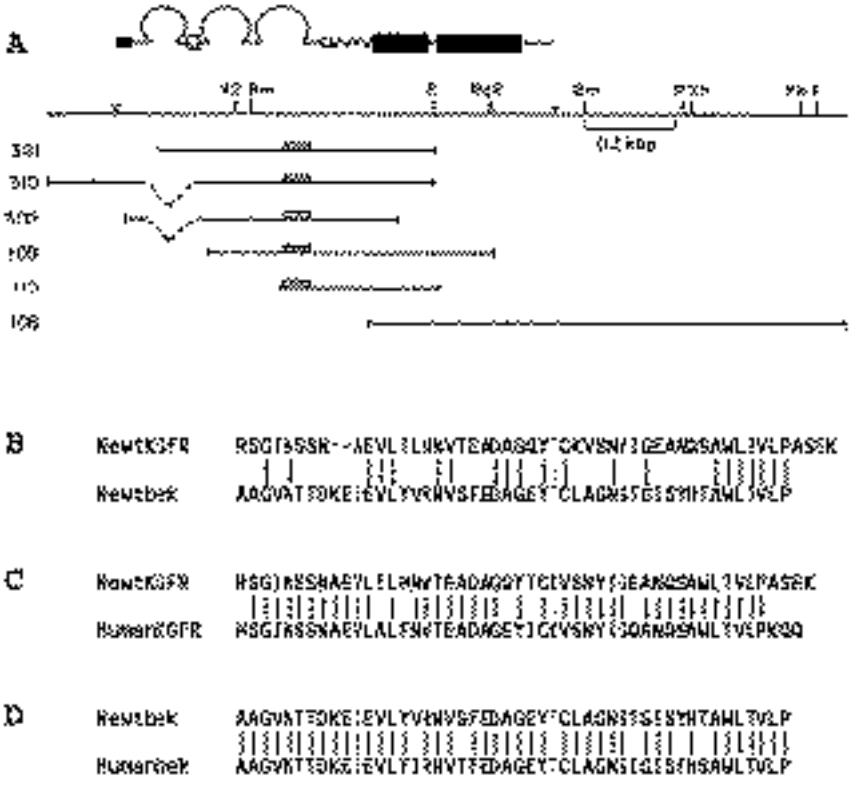

Fig. 1. Representation of NvFGFR2 cDNA clones and comparison of the two amino acid sequences encoded by the second half of the last Ig-like domain. (A) The receptor molecule is graphically represented above the cDNA clones. The major restriction sites are shown between the graphic model and the cDNA clones (Bm, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; H2, *Hin*dII; P, *Pst*I; Xb, *Xba*I; Xh, *Xho*I). The model is drawn to linear scale in relation to the cDNA sequence. The open and closed triangles represent the initiation and termination codons, respectively. The three loop structures represent the immunoglobulin (Ig)-like extracellular domains and the stippled box between the first and second Ig-like domains represents the acidic domain. The open box followed by the wavy line represents the transmembrane and juxtamembrane domains and the closed box represents the tyrosine kinase domain bisected by the kinase insert. The thickened line represents the hydrophobic signal peptide. The hatched box on the cDNA clones represents sequence that codes for the KGFR isoform (IIIb) whereas the cross-hatched box on clone 110 represents the *bek*-like (IIIc) isoform. The clones that are made contiguous with dashed lines represent cDNAs in which the first Ig-like domain is spliced out. Clone 301 represents the three loop form of newt FGFR2. (B) Alignment of the amino acid sequences encoded by the newt KGFR (clones 109, 301, 302 and 310) and *bek* (clone 110) cDNA clones. (C) Alignment of the amino acid sequences of newt and human KGFR. (D) Alignment of the amino acid sequences of newt and human *bek*. The human sequences were obtained from previously published data (Miki et al., 1991, and Dionne et al., 1990).

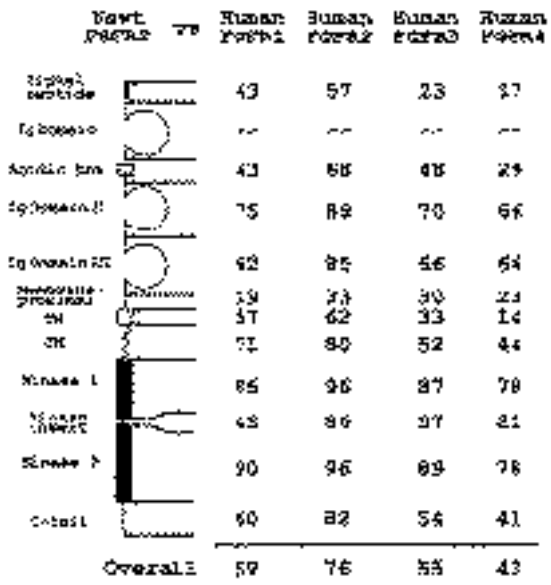

Fig. 2. Amino acid sequence similarity between newt FGFR2 and the four members of human FGF receptor family. The KGFR variant of newt FGFR2 was used for comparison. Similarly, the KGFR variant of human KGFR2 (Miki et al., 1992) was used for comparison. Other known human FGF receptor sequences including FGFR1 (Dionne et al., 1990), FGFR3 (Keegan et al., 1991) and FGFR4 (Partanen et al., 1991) were also used for comparison.

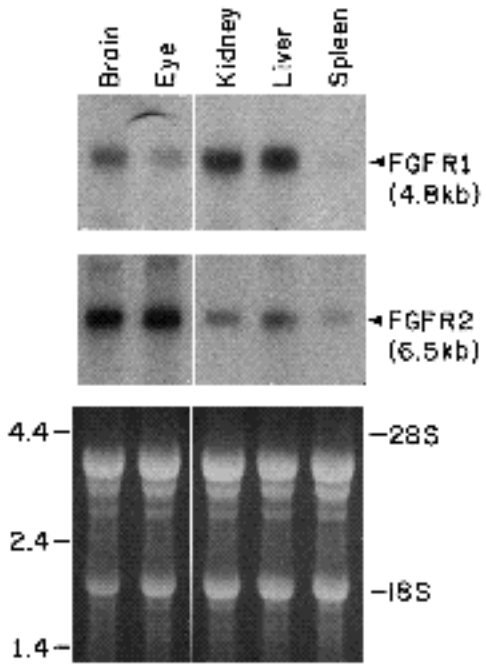

Fig. 3. Northern blot analysis of newt tissues. Total RNA from newt brain, eye, kidney, liver and spleen was hybridized to ^{32}P-labeled antisense riboprobes used in the in situ hybridizations. A single hybridizing band was observed using either the FGFR1 or FGFR2 riboprobes. The ethidium bromide-stained agarose gel is shown to indicate loading the same amount of the RNA in each lane. The neural derived brain and eye express higher levels of the 6.5 kb FGFR2 mRNA while the mesodermally derived kidney and liver express higher levels of the 4.8 kb FGFR1 mRNA. The markers on the left are in kilobases (kb) and the 28S and 18S markers on the right are where the human large and small rRNA ran on the gel. Note that the newt large rRNA runs faster than the human 28S rRNA in the gel.

sequence similarity of newt FGFR2 with the four human FGF receptors are 59, 76, 55 and 43%, respectively. Similarly, the amino acid identity of clone 102 with the four human FGF receptors are 90, 81, 77 and 69%, respectively, indicating that this cDNA encodes the newt FGFR1.

Northern hybridization

As a first step toward determining the FGFR expression pattern and specificity of the riboprobes, northern analysis on various newt tissues was carried out. The FGFR1 and FGFR2 antisense riboprobes used for the in situ hybridizations were hybridized to newt brain, eye, kidney, liver and spleen RNA. A single band of 4.8 kb was observed in these tissues with the FGFR1 riboprobe with the kidney and liver showing the highest intensity of signal. When the same filter was stripped and hybridized to an FGFR2 riboprobe, a single band of 6.5 kb was observed with the brain and eye showing the highest level of hybridization (Fig. 3). Since FGFR1 and FGFR2 are more similar to each other than each one of them is to the other FGF receptors or to other tyrosine kinase receptors (Johnson and Williams, 1993; also see Fig. 2), and the sizes of the FGFR1 and FGFR2 transcripts are distinctly different, it is not likely that these probes detected other FGFR-like sequences. However, we do not know whether they represent the two-loop or three-loop forms and, in the case of FGFR2, whether the hybridizing band represent the *bek* or KGFR variant. Since the two-loop and three-loop forms differ by 200 nucleotides in size, it is not possible to distinguish these two different messages based on gel mobility. Similarly, it is not possible to distinguish the *bek* and KGFR messages by size alone because they differ in size by six nucleotides.

In situ hybridizations

Cryosections of regenerating newt limbs of different stages (staged according to Iten and Bryant, 1973) were hybridized to both sense and antisense ^{35}S-labeled riboprobes, washed and exposed to photographic emulsion. The slides were developed, stained with hematoxylin and counter-stained with eosin, and examined under both light- and dark-field microscopy. The fragment used to generate the FGFR2 riboprobes was a 306 bp *Bbs*I-*Bam*HI fragment containing the second Ig-like extracellular domains (nt. 465-770). This antisense riboprobe recognizes all known isoforms, generated by alternative splicing, of newt FGFR2. The newt FGFR1-specific riboprobe represents the last 73 amino acids of the carboxyl terminus and 80 nucleotides of the 3′-untranslated sequence.

At the preblastema stage of regeneration there were two FGFR2-hybridizing regions observed in the regenerate (Fig. 4A). The first region was in the wound epithelium. At this stage, the wound epithelium was 5-10 cell layers thick and the hybridization was seen in the basal cells adjacent to the

underlying mesenchyme but not in the outer layers (Fig. 4B). This hybridization did not extend into the limb epidermis and thus was specific for the wound epithelium. Additional hybridization was also observed in the cells of the periosteum (Fig. 4C). At this stage no signal is detected over the dedifferentiating mesenchyme cells (Fig. 4A). The hybridization signals are specific to the FGFR2, since the sense probe revealed negligible signals in the wound epithelium and periosteum (data not shown).

The early bud blastema showed three distinct hybridization areas. The first was the basal layer of the wound epithelium (Fig. 4D) as seen in the preblastema stage. At a higher magnification, it was apparent that the hybridization signal decreases abruptly at the amputation boundary (Fig. 4E). The second was in the blastema mesenchyme but was largely restricted to cells in the core of the blastema adjacent to the ends of the bisected bones (Fig. 4D). In contrast, little hybridization was seen in the more distal mesenchymal cells adjacent to the wound epithelium. The third area of hybridization was in the periosteum, observed in the stump (Fig. 4D) as in the preblastema stage regenerate.

The mid-bud blastema showed the same pattern of hybridization to FGFR2 as the early bud blastema but a greater intensity of signal was seen in the blastema mesenchyme, probably due in part to the increased cell number at this stage of regeneration. The hybridization signal seen in the basal cell layer of the wound epithelium remained unchanged (data not shown). By contrast, the FGFR1 hybridization pattern was homogeneously distributed in the blastema mesenchyme throughout the proliferative stages of regeneration spanning from the early blastema stage until the mid-bud stage (Fig. 4F). Most notably, there was no FGFR1 hybridization in the wound epithelium unlike that observed for FGFR2 (Fig. 4F).

As growth slowed and differentiation began, a different pattern of hybridization to FGFR2 emerged. Hybridization was now concentrated in the condensing cartilage and followed the pattern of the forming digits. The wound epithelial expression of FGFR2 detected early in the preblastema stage, which lasted till the mid-bud stage, was no longer observed (Fig. 4G,H). As differentiation continued into the digit stages of regeneration, the pattern of hybridization to FGFR2 became more restricted to the perichondrial regions of the forming digits and metacarpals, with less intense hybridization remaining in the preossified cartilage of the regenerate (Fig. 4I). At this stage, the hybridization pattern of FGFR1 is very similar to that of FGFR2 (data not shown).

DISCUSSION

Our results show that the newt FGF receptors 1 and 2 (NvFGFR1 and NvFGFR2) are both expressed in the blastema during forelimb regeneration. However, the temporal and spatial expression patterns of these receptors are different, indicating that the two receptors may have different roles. FGFR1 expression is homogeneously distributed throughout the blastema mesenchyme. FGFR2 is expressed in the core of the blastema adjacent to and surrounding the bisected bone, as well as in the basal layer of cells in the wound epithelium during the stages of regeneration associated with growth and blastema cell proliferation. During differentiation stages, FGFR2 expression is predominantly seen in the condensing cartilage of the early digit stage and in the perichondrium of the late digit stage. It has been shown in other species that FGFR1 is a receptor for aFGF, bFGF and FGF-4 (Dionne et al., 1990; Johnson et al., 1991; Mansukhani et al., 1990) and that FGFR2 is a receptor for aFGF and either bFGF or KGF, depending on the isoform (Dionne et al., 1990; Mansukhani et al., 1992; Miki et al., 1992; Yayon et al., 1992). We have transfected the newt KGFR cDNA into Chinese hamster ovary cells and the expressed protein exhibits binding affinities similar to those of the human KGFR. As such, the newt KGFR-expressing CHO cells bind to ^{125}I-aFGF, and this binding can be competed by both aFGF and KGF but not by bFGF (K. Patrie, unpublished results). These results suggest that the newt KGFR not only shares sequence homology but also functional similarity with KGFR of other species. Thus, blastema cells could theoretically respond to different FGF ligands and it is important now to consider the cellular source of various members of the FGF family. In Fig. 5 we summarize the in situ hybridization results of FGFR1 and FGFR2 in the blastema, and provide a testable model to account for a multiplicity of FGFs and FGFRs in regenerating limbs. This model is established based on our in situ hybridization results for the FGFRs shown here and on other previously published results for the FGFs.

The relative levels of FGF receptor mRNAs in the different newt tissues are also different as shown by northern analyses. FGFR1 is expressed at higher levels in the mesodermally derived kidney and liver than in the neurally derived brain and eye. There is no expression of this receptor in the spleen. FGFR2, in contrast, is expressed at higher levels in the brain and eye than in the kidney and liver and expression is detectable in the spleen. FGFR1 expression seems to correlate strongly with mesodermal tissues as seen in the kidney and liver by northern hybridization (Fig. 3) and in the blastema mesenchyme observed by in situ hybridization (Fig. 4F). FGFR2, in contrast, is shown to be expressed at high levels in nervous tissue by northern hybridization. Perhaps the expression of FGFR2 in the blastema, as seen by in situ hybridization, is in response to the neurotrophic factor(s) released into the blastema by nerves.

In developing mouse limbs, FGF-4 is expressed in the apical ectodermal ridge (AER) and, since FGF-4 has a signal peptide and can therefore be secreted, it was suggested that the target of FGF-4 is the limb mesenchyme (Niswander and Martin, 1992). It is likely that the AER and the wound epithelium carry out similar functions, i.e. epithelial/mesenchymal interactions, essential for limb bud development and blastema outgrowth, respectively (Muneoka and Sassoon, 1992). Thus the wound epithelium may also release FGF-4 into the mesenchyme where it interacts with FGFR1 and stimulates mesenchyme growth (Fig. 5). It is noted that FGF-4 has not been identified in newts. Moreover, it is noted that an XeFGF isolated from *Xenopus* embryos shares equal amino acid identity to both FGF-4 and FGF-6 but FGF-4 and FGF-6 have not been found in this organism (Issacs et al., 1992). Therefore, XeFGF may be the evolutionary precursor for the mammalian FGF-4 and FGF-

6. Thus, the newt FGF-4 here should be considered the FGF-4/FGF-6/XeFGF cognate.

Another possible source of one or more FGFs is the nerve. It has long been known that nerves are essential for limb regeneration (Singer, 1952) but the identity of the putative neurotrophic factor(s) is not yet known (Carlone and Mescher, 1985). It has been shown that a crude FGF preparation exhibited some mitogenicity when infused into den-

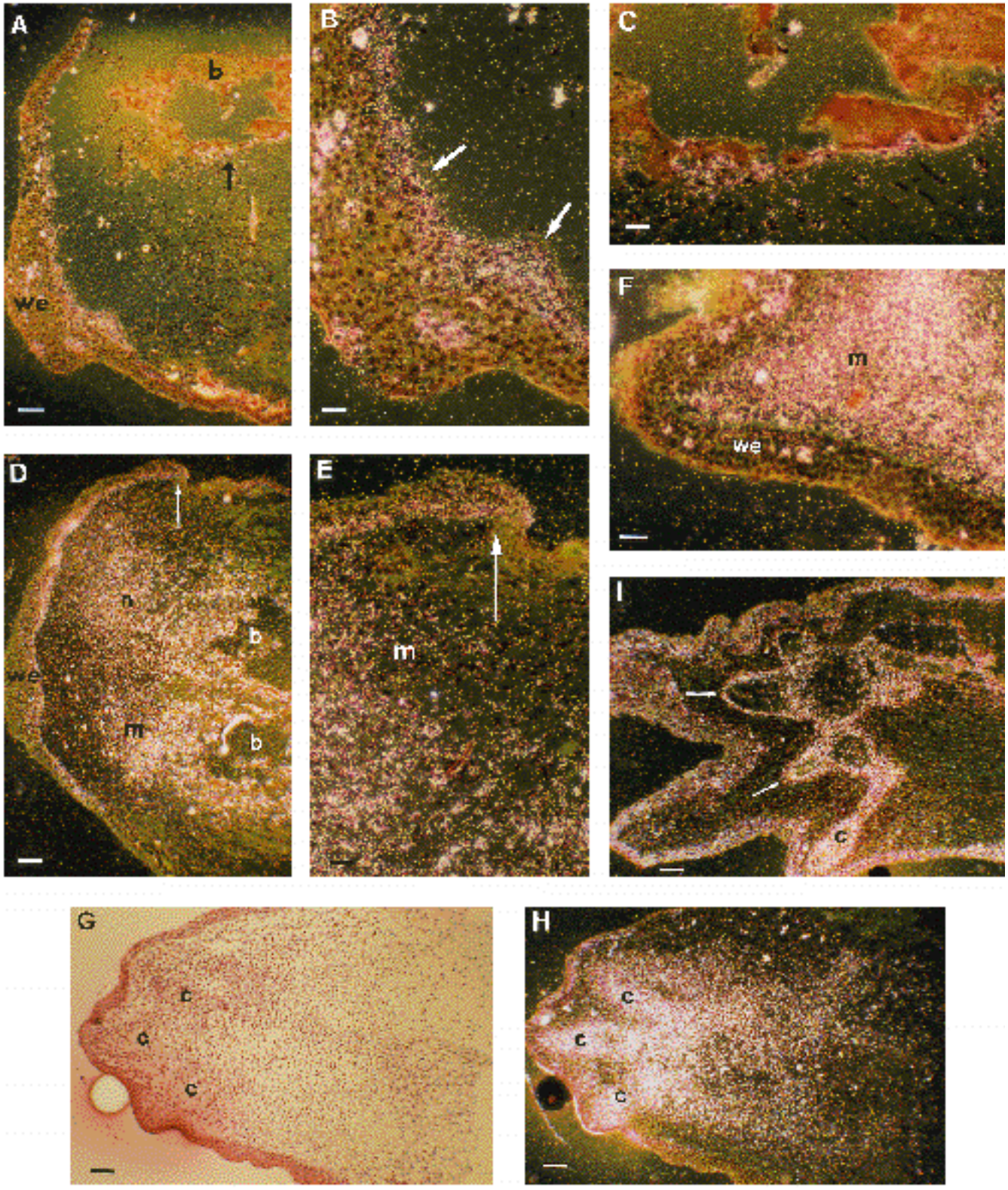

Fig. 4

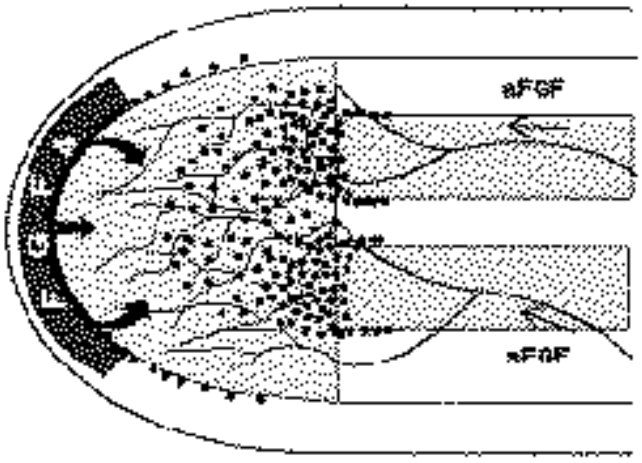

Fig. 5. A model suggesting interactions of FGFs and their receptors in regenerating limbs. The in situ hybridization results of FGFR1 and FGFR2 in regenerating limbs are summarized diagramatically. FGFR1 is expressed by all blastema mesenchyme cells (light grey). FGFR2 is expressed by cells of the cartilage lineage and by basal cells of the wound epithelium (circles). Neurons synthesize aFGF which is transported by axons (thick lines) to the blastema (as indicated by thin arrows) where it interacts with FGFR1 or FGFR2 on mesenchyme cells to stimulate their division. FGF-4 (representing the newt cognat of XeFGF/FGF-4/FGF-6) is produced by wound epithelium (dark grey) which also interacts with FGFR1 on mesenchyme cells (as indicated by thick arrows) to stimulate cell division further and/or to prevent differentiation. One of the FGFs interacts with FGFR2 to restrict cells to the cartilage lineage and/or to cause the FGFR2-expressing cells to form cartilage. FGFR2 expressed by the wound epithelium is involved in either FGF stimulation of wound epithelial cell cycling and/or in a specific function of the wound epithelium.

ervated newt limb stumps (Mescher and Gospodarowicz, 1979; Gospodarowicz and Mescher, 1980). In mice, it was shown that aFGF mRNA is present in ganglia by in situ hybridization and that aFGF is present within peripheral nerves closely associated with the cytoplasmic side of the axonal membranes by immunohistochemical studies (Elde et al., 1991). Moreover, aFGF is present in the blastema during limb regeneration in axolotls (Boilly et al., 1991). Thus, it is possible that during blastema development aFGF is released from nerves into the blastema where it can react with mesenchymal cells expressing FGFR1, and with the cells that are expressing FGFR2 in the core of the blastema adjacent to the bisected bone. It may also interact with the cells within the wound epithelium that are expressing FGFR2 (Fig. 5). A recent demonstration of multiple tissue-specific promoters in the aFGF gene (Myers et al., 1993) lend credence to the theory that the aFGF gene may be equipped with the flexibility to respond to various developmental or traumatic situations.

The expression pattern of FGFR2 raises the possibility that this receptor is restricted to the cartilage lineage. The expression of FGFR2 in the blastema is seen initially in those cells closely associated with the bisected ends of the radius and ulna. Subsequently, FGFR2 expression is associated with mesenchymal cells condensing to form the skeletal primordia. Utilizing triploidy and thymidine-labeled grafts of either cartilage or muscle, Steen (1968) showed that, in axolotls, the cartilage lineage is very stable; most cartilage cells give rise to blastema cells that then redifferentiate back into cartilage. Steen (1968) and others have also shown that connective tissue cells can give rise to cartilage (reviewed in Bryant and Gardiner, 1992). While lineage studies have not been done in newts, it seems reasonable to suggest that periosteal cells contribute to the blastema cartilage lineage. This view is supported by our observation in unamputated limbs (data not shown) and in the limb stump (Fig. 4C) that FGFR2 is expressed in periosteal cells and in cartilage of the epiphyses of the radius and ulna and in the autopodium. Thus, chondrocytes and periosteal cells already expressing FGFR2 may be recruited into the blastema early in regeneration and then largely maintained in the cartilage lineage. In this regard, Gospodarowicz and Mescher (1980) showed that FGF preparations can stimulate chondroblast proliferation. Also, aFGF has been isolated from bovine scapular cartilage (Sullivan and Klagsbrun, 1985) and bone (Hauschka et al., 1986). Perhaps in regeneration, aFGF interacts with FGFR2 to stimulate chondroblast proliferation and/or to maintain the cartilage lineage.

Fig. 4. Micrographs illustrating the localization of FGFR1 and FGFR2 mRNA in various stages of blastema development. Antisense ^{35}S-labeled FGFR2 riboprobes were hybridized to cryosections of a day 10 preblastema stage regenerate and visualized by dark-field microscopy. (A) Hybridization signal was detected in the basal layer of the wound epithelium (we), which is shown at higher magnification in B, and to the cells of the periosteum (black arrows) of the bisected bone (b), shown in C under higher magnification. At this stage no signal is seen over the dedifferentiating mesenchyme cells. A dark-field micrograph showing the hybridization pattern of the antisense FGFR2 riboprobe to an early bud blastema at (D) low and (E) high magnification. The low-magnification micrograph in D shows FGFR2 transcripts in the cells of the basal layer of the wound epithelium (we) which decrease dramatically in the stump epidermis (white arrow marks border of wound epithelium and epidermis). FGFR2 mRNA is also seen in the mesenchymal cells (m) of the blastema closely associated with the bisected bones (b). The high magnification of E shows that the FGFR2 mRNA is restricted to the wound epithelium and mesenchyme (m) and decreases abruptly at the amputation level (white arrow). e, stump epidermis. (F) A micrograph illustrating hybridization of the FGFR1 antisense riboprobe in a mid bud blastema, visualized by dark-field microscopy. This micrograph shows the restricted localization of FGFR1 mRNA to the blastema mesenchyme (m) and its distinct absence from the wound epithelium (we) and stump tissue. The white dashed line indicates the level of amputation. Light-field (G) and dark-field (H) micrographs of an early digit stage regenerate hybridized to the antisense FGFR2 riboprobe. The expression pattern is very specific to the cells of the condensing cartilage (c). (I) Dark-field micrographs showing the location of FGFR2 mRNA in a late digit regenerate and the specificity of the FGFR2 riboprobe. The micrograph shows FGFR2 expression associated with the condensing cartilage (c) and the periosteum (white arrows). A,D,G,H,I, Bar, 120 μm. B,C,E,F, Bar, 50 μm

The results suggest that FGFR2 may also play a role in specific functions of the wound epithelium and/or in wound epithelial development into skin. We show here that FGFR2 expression is observed in the basal layer of the wound epithelium during preblastema stages and this expression persists until differentiation stages. In the developing mouse, the entire body ectoderm, including the limb bud ectoderm, expresses FGFR2 (Orr-Urtreger et al., 1991; Peters et al., 1992) suggesting that FGFR2 is important for normal development of skin. In a wound healing study, KGF was shown to be induced 160-fold one day after skin injury (Werner et al., 1992). This large induction was unique within the FGF family, since mRNA levels of aFGF, bFGF and FGF-5 were induced only 2- to 10-fold during wound healing, and there was no expression of FGF-3, FGF-4 and FGF-6 detected in normal and wounded skin. In situ hybridization showed expression of KGF in the dermis while FGFR2 was predominantly expressed in the epidermis (Werner et al., 1992). Whether the epidermal FGFR2 is the KGFR or the *bek* variant remains to be determined. We found that the spatial and temporal patterns of expression of FGFR1 and FGFR2 (Fig. 4) during limb regeneration are reminiscent of those seen in embryonic limb development (Orr-Urtreger et al., 1991; Peters et al., 1992). Furthermore, at the initial stage of blastema formation, the distribution of the FGF receptors in the wound epithelium duplicate those seen in the back skin wounding model (Werner et al., 1992).

The wound epithelium is a necessary component of the regenerate (Singer and Salpeter, 1961) and has been shown to express a number of molecules not expressed by skin epidermis, including the antigens designated WE3 (Tassava et al., 1986), WE4 (Castilla and Tassava, 1992), MT1/tenascin (Onda et al., 1991), and MT2 (Klatt et al., 1992). It is not inconceivable that the interaction of FGFR2 with its ligand is somehow involved with the synthesis and/or function of these wound epithelial antigens. Finally, while innervation of the wound epithelium is not essential for regeneration (Sidman and Singer, 1960), an FGF-like neurotrophic factor released from nerves may nevertheless interact with FGFR2 in the wound epithelium, either in stimulating proliferation or in establishing a functional wound epithelium.

In summary, the results show that heterogeneity exists in the expression patterns of FGFR1 and FGFR2 during limb regeneration in newts. It remains to be seen whether the FGFR2 present in the regenerating amphibian limb is the KGFR or *bek* variant. Similarly, it will be of interest to identify which splice variant of FGFR1 is present in the blastema mesenchyme. It is also likely that similar heterogeneity exists in FGF ligands. With the establishment of existence of FGFRs in various cell types of the regenerating limb, the source of these ligands within the blastema now warrants further investigation.

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