

Spatially restricted expression of fibroblast growth factor receptor-2 during *Xenopus* development

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Summary

The fibroblast growth factors (FGFs) play a role in *Xenopus laevis* embryonic development, particularly in the induction of ventral-type mesoderm. We have isolated a full-length cDNA from *Xenopus* that we have designated *Xenopus* fibroblast growth factor receptor-2 (XFGFR-2), with significant amino acid sequence similarity to the previously described *bek* gene (FGFR-2). We expressed the XFGFR-2 cDNA in COS1 cells and showed that it functions as an FGF receptor by binding radiolabeled FGF-2. RNA gel blot analysis demonstrates that unlike *Xenopus* fibroblast growth factor receptor-1 (XFGFR-1), XFGFR-2 mRNA expression begins during gastrulation and continues through early tadpole

stages. Whole-mount in situ hybridization demonstrates that XFGFR-2 mRNA is localized to the anterior neural plate in early neurula stage embryos. Later in development, XFGFR-2 expression is found in the eye anlagen, midbrain-hindbrain boundary and the otic vesicle. In addition, XFGFR-2 transcripts are expressed in animal caps in a manner that is independent of mesoderm-inducing factors. These results indicate that XFGFR-2 may have a role in development that is distinct from that of XFGFR-1.

Key words: *Xenopus*, FGF receptor, gene expression, tyrosine kinase.

Introduction

Recent experimental evidence indicates that the protein tyrosine kinases (PTKs) have a prominent role in developmental processes including migration, proliferation and differentiation. Many PTKs have been shown to be expressed at high levels during embryonic development, often in a localized manner and often at much higher levels than are expressed in adults (Pawson and Bernstein, 1990). Normal function of certain PTKs has been shown to be crucial to normal development (Pawson and Bernstein, 1990). Evidence suggests that when normal PTK function is perturbed during embryogenesis, major phenotypic changes can result (Pawson and Bernstein, 1990). To understand how PTKs regulate developmental events, the identification and characterization of PTKs expressed in embryos is important.

The fibroblast growth factors (FGFs) are polypeptides that are involved in many important developmental processes including angiogenesis, chemotaxis, proliferation, neuronal survival and mesoderm induction of *Xenopus* embryonic ectoderm (Burgess and Maciag, 1989). The FGF family now consists of at least seven members (Abraham et al., 1986; Jaye et al., 1986; Bovi et al., 1987; Yoshida et al., 1987; Dickson and Peters, 1987; Zhan et al., 1988; Marics et al., 1989; Finch et al., 1989). The two most characterized members of the family are FGF-1 and FGF-2

(Burgess and Maciag, 1989). Several members of the family are expressed during embryonic development and exhibit both unique and overlapping spatiotemporal patterns of expression (Hebert et al., 1990). Although many developmental roles have been proposed for members of the FGF family, none have been established experimentally. However, several members of the FGF family have been shown to induce mesoderm formation in *Xenopus* ectodermal explants (Kimelman and Kirschner, 1987; Slack et al., 1987; Paterno et al., 1989). In addition, both FGF-1 and FGF-2 have been shown to be present in eggs and early embryos and may serve some role in the earliest inductive events in *Xenopus* development (Kimelman et al., 1986; Slack and Isaacs, 1989; Shiurba et al., 1991).

The fibroblast growth factor receptors (FGFRs) may also be grouped into a gene family, and evidence that these genes may perform tissue-specific as well as developmental-specific functions is accumulating. The receptor for FGF-2 was affinity purified from chick embryos, sequenced and subsequently cloned (Lee et al., 1989). The chicken bFGF receptor was shown to be homologous to the human *fms*-like gene (*flg*) tyrosine kinase (Pasquale and Singer, 1989; Lee et al., 1989; Ruta et al., 1988). A second FGF receptor cDNA called *bek* has been cloned from human (Dionne et al., 1990; Hattori et al., 1990), mouse (Raz et al., 1991; Kornbluth et al., 1988) and chicken (Pasquale, 1990) cDNAs. Both *flg* (FGFR-1) and *bek* (FGFR-2) have

been shown to bind FGF-1 and FGF-2 with similar affinities (Dionne et al., 1990). An additional tyrosine kinase gene belonging to the FGFR family (*cek2*) has been cloned from a chicken cDNA library (Pasquale, 1990). In addition, two more FGFR cDNAs, called FGFR-3 (Keegan et al., 1991) and FGFR-4 (Partanen et al., 1991) have been cloned from K562 erythroleukemia cells. The chicken gene *cek2* seems to be the homologue of the human FGFR-3 gene (Pasquale, 1990; Partanen et al., 1991). FGFR-3 binds FGF-1 and FGF-2, whereas FGFR-4 binds FGF-1 but not FGF-2; thus, there seems to be some ligand-specific receptors among members of the FGFR family (Keegan et al., 1991; Partanen et al., 1991). Finally, a fifth member of the FGFR family has been cloned from a human keratinocyte library and has been designated *flg-2* (FGFR-5) (Avivi et al., 1991). *Flg-2* is most closely related to FGFR-3 (Jaye et al., 1992). Structural features common to members of the FGFR family include a signal peptide, two or three immunoglobulin-like loops in the extracellular domain and a highly conserved tyrosine kinase domain split by a short kinase insert sequence. The embryonic expression patterns of the FGFRs are also distinct (Partanen et al., 1991). Furthermore, several members of the FGFR family have been shown to exist in multiple forms as the result of alternative splicing mechanisms (Hou et al., 1991; Friesel and Dawid, 1991; Johnson et al., 1990; Miki et al., 1992; Reid et al., 1990; Werner et al., 1992).

The importance of FGFR signaling in embryonic development was recently demonstrated by Amaya et al. (1991). These investigators demonstrated that expression in *Xenopus* embryos of an FGFR lacking the tyrosine kinase domain abolished wild-type receptor function. Explanted animal pole ectoderm derived from embryos expressing the truncated receptor failed to form mesoderm in response to FGF-2. This effect could be rescued by forced overexpression of the wild-type receptor. These data clearly establish a role for the FGFs and their receptors in embryonic pattern formation; however, the mechanisms by which this occurs remains to be elucidated.

In this report, we describe the isolation of a cDNA clone for *Xenopus* FGFR-2. Analysis of the temporal and spatial patterns of XFGFR-2 mRNA expression indicate that its function may be independent of mesoderm induction and may thus have a role in *Xenopus* development that is distinct from that of XFGFR-1.

Materials and methods

Isolation of XFGFR-2 cDNA clones

We have previously described the isolation and cloning of the *Xenopus* homologue of FGFR-1 by screening a Zap library of *Xenopus* XTC cell cDNA with synthetic degenerate oligonucleotides corresponding to a conserved region of the intracellular domain of FGFR-1 (Friesel and Dawid, 1991). During this screening a 2.9 kb clone was isolated that was unique by restriction enzyme mapping. Sequence analysis showed this clone to encode a deduced protein with homology to FGFR-2. The 2.9 kb clone lacked some 3' sequences encoding the carboxy-terminal tail of the deduced protein. To obtain a full-length clone containing all of the 3' coding region, we employed the RACE-PCR protocol as described by Frohman et al. (1988). 1 µg of poly(A)⁺ RNA from

Xenopus ovary was reverse transcribed with 0.5 µg (dT)₁₇-adaptor and 200 U of Moloney murine leukemia virus reverse transcriptase (Bethesda Research Laboratories) for 2 hours at 42°C. The cDNA was diluted to 0.5 ml with TE (10 mM Tris-HCl, pH 7.5/1 mM EDTA). 10 µl of cDNA was added together with 25 pmol of outer adapter primer (R_O) and 25 pmol of gene-specific primer (GSP1) corresponding to the kinase insert domain (5'-TTGAGATGGAGTACTCATTCG-3') in a 50 µl PCR reaction mixture (1× *Taq* polymerase buffer (Perkin-Elmer-Cetus) and dNTPs at 2 mM each) were denatured at 95°C for 5 minutes and then cooled to 72°C followed by addition of 2.5 U of *Taq* DNA polymerase (Perkin-Elmer-Cetus) and overlaid with 50 µl of mineral oil. The reaction was annealed at 50°C for 5 minutes followed by extension at 72°C for 10 minutes. The DNA thermal cycler (Perkin-Elmer-Cetus) step program was as follows: 94°C, 1 minute; 56°C, 1 minute; 72°C, 3 minute for 40 cycles. One-fifth of the polymerase chain reaction (PCR) product was subjected to electrophoresis on a 1.5% agarose gel and a major band of 0.9 kb was identified by ethidium bromide staining. The band was excised from the gel and purified with a GeneClean kit (Bio101). The purified fragment was subjected to another round of PCR amplification with primers GSP1 and inner adapter primer (R_I) essentially as described for the first round of amplification. A 0.9 kb fragment from the second round of amplification was purified from a 1.5% agarose gel with GeneClean and cloned into the pCR1000 vector (Invitrogen) for amplification and sequencing.

DNA sequence analysis

Nucleotide sequencing was performed on double-stranded templates by the dideoxy-chain termination method (Sanger et al., 1977; Korneluk et al., 1985) using a Sequenase kit (United States Biochemicals) and [³⁵S]dATP (>1000 Ci/mmol; Amersham). For sequencing clones >2 kb, sets of nested deletions were prepared with exonuclease III and S1 nuclease (Erase-a-base; Promega). Second-strand sequencing was performed using synthetic oligonucleotide primers and Sequenase. Nucleic acid sequences were aligned and analyzed using PCGENE (Intelligence).

Xenopus eggs and embryos

Eggs were obtained from adult female frogs (*Xenopus* 1) that had been induced to spawn by injection of 800 U of human chorionic gonadotropin (Sigma). Eggs were fertilized in vitro with sperm from minced testes. Fertilized eggs were allowed to undergo cortical rotation, were dejellied in 2% cysteine and rinsed in dechlorinated water. Embryos were allowed to develop in 0.1× modified Ringer's medium (MMR) at 19–22°C and staged according to Nieukoop and Faber (1967). Microdissection of animal caps and incubation with growth factors were essentially as described (Friesel and Dawid, 1991; Smith et al., 1988).

RNA gel blot analysis

Total RNA was prepared from *Xenopus* eggs and embryos at different stages of development and from ectodermal explants essentially as described by Chomczynski and Sacchi (1987). RNA was denatured, separated by electrophoresis through 1.0% agarose gels containing 2.2 M formaldehyde, electroblotted onto nylon membranes (Nytran, Schleicher & Schuell) and cross-linked with ultraviolet light (Stratalinker, Stratagene). Probes for hybridization were labeled to high specific activity with [³²P]-dCTP (3000 Ci/mmol, Amersham) using a random primer labeling kit (Boehringer Mannheim). The XFGFR-2 probe used in these studies was a 1.8 kb restriction fragment encompassing the extracellular domain as well as some 5' untranslated sequences. In some experiments, blots were stripped (Feinberg and Vogelstein, 1983)

and reprobed with a 1.3 kb restriction fragment of XFGFR-1 spanning the coding region of the extracellular domain of this receptor. Hybridizations were carried out by the method of Church and Gilbert (1984) in buffer containing 0.5 M sodium phosphate (pH 7.0), 7% sodium dodecyl sulfate (SDS), 1% bovine serum albumin (BSA) and 10 mM EDTA at 65°C for 16 hours. Filters were washed in 0.2× SSPE at 65°C for 1 hour and exposed to X-ray film (XAR-5, Kodak) with intensifying screen (Cronex, DuPont) at -70°C. To confirm that equivalent amounts of RNA were loaded, blots were rehybridized to a *Xenopus* 18S rRNA oligonucleotide probe as previously described (Friesel and Dawid, 1991).

Expression constructs

The complete open-reading frame of XFGFR-2 with all of the 5 and 3 untranslated sequences removed was generated by PCR. The 2.4 kb PCR fragment was blunt ended using T4 DNA polymerase and phosphorylated with T4 polynucleotide kinase. The fragment was ligated into dephosphorylated, *EcoRV*-digested pcDNAIneo (Invitrogen). The ligation was transformed into competent *E. coli*. Positive clones were mapped by restriction enzyme digestion to confirm their orientation relative to the cytomegalovirus promoter of the vector. One of these clones, designated pcDNAIneo-XFR2.6, was selected for expression studies.

DNA transfection into COS1 cells

COS1 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Irvine Scientific) supplemented with 10% fetal bovine serum (FBS) (GIBCO). For transient transfection studies, COS1 cells were plated at 5×10^5 cells per 100 mm dish. 16 hours later, pcDNAIneo-XFR2.6 or pcDNAIneo as a control plasmid were transfected into cells by the DEAE-dextran method (Maniatis et al., 1982). Following transfection, cells were incubated in DMEM-10% FBS for 2-3 days at 37°C.

FGF receptor cross-linking

Monolayers of transfected COS1 cells were washed once with cold DMEM containing 50 mM N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid (Hepes), pH 7.4, 0.1% bovine serum albumin (BSA) and 10 U/ml heparin (Upjohn) (binding buffer). Cells were incubated at 4°C for 1 hour in the presence of binding buffer containing 10 ng/ml ¹²⁵I-FGF-2 in the presence or absence of a 200-fold excess of unlabeled FGF-2 or FGF-1. The cells were then washed twice with cold binding buffer, once with cold phosphate-buffered saline (PBS) and incubated for 20 minutes at 4°C in PBS containing 0.3 mM disuccinimidyl suberate (DSS). The cells were washed once with PBS containing 0.1 M Tris, pH 7.4, scraped from the dish, pelleted and extracted in lysis buffer (20 mM Hepes, pH 7.4, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM MgCl, 1.0 mM EGTA, 1 mM PMSF, 10 µg/ml aprotinin) and centrifuged for 10 minutes at 10,000 g. Supernatants were mixed with an equal volume of 2× SDS sample buffer and analyzed on either 7.5 or 9% SDS-polyacrylamide gels.

Whole-mount in situ hybridization

In situ hybridization experiments were performed following the methods described by Hemmati-Brivanlou et al. (1990) and Harland (1991). Embryos derived from albino females were used in all experiments. Digoxigenin-labeled sense and antisense RNAs were prepared by in vitro transcription of linearized plasmids containing the extracellular domain of XFGFR-2 using an RNA labeling kit (Boehringer Mannheim). Hybridized digoxigenin-containing RNAs were visualized with anti-digoxigenin antibodies (Fab fragment) conjugated to alkaline phosphatase (Boehringer Mannheim).

Results

Cloning and sequence analysis of *Xenopus* FGFR-2 cDNA

We have previously described the cloning and expression of XFGFR-1, which was obtained by screening a *Xenopus* cDNA library with degenerate oligonucleotide probes corresponding to a conserved region of the intracellular domain of the human *flg* gene (FGFR-1) (Friesel and Dawid, 1991). Several clones were isolated and shown to encode the *Xenopus* homologue of FGFR-1 (*flg*). Restriction enzyme mapping of one of the isolated clones showed that it was distinct from XFGFR-1. Nucleotide sequence analysis demonstrated that this clone contained most of the coding region of another member of the FGFR family, with the highest degree of homology being to FGFR-2 (*bek*) (Dionne et al., 1990). By comparison to the human and chicken FGFR-2 sequences, this clone lacked the cytoplasmic tail and did not contain a termination codon. To obtain clones containing the 3' end of this gene, we employed the 3' RACE-PCR protocol originally described by Frohman et al. (1988). Following RACE-PCR amplification, a 900 bp fragment was subcloned and sequenced. This cDNA encoded the deduced amino acid sequences for the cytoplasmic tail as well as an in-frame termination codon and 3' untranslated sequences.

The entire deduced amino acid sequence of XFGFR-2 is shown in comparison to XFGFR-1 in Fig. 1. The open-reading frame of XFGFR-2 encodes a protein of 813 amino acids with a calculated molecular mass of 91,598. The *Xenopus* FGFR-2 has several features in common with other members of the FGFR family. The initiation methionine is flanked by a satisfactory Kozak consensus sequence (not shown) followed by a putative hydrophobic signal peptide. The extracellular domain is made up of three immunoglobulin-like domains (Lee et al., 1989). There are seven consensus sequences for asparagine-linked glycosylation in the extracellular domain. Also conserved in XFGFR-2 is a unique stretch of acidic residues between the first and second immunoglobulin-like domains. A hydrophobic transmembrane domain joins the extracellular domain to the intracellular tyrosine kinase domain. The tyrosine kinase domain contains an ATP-binding domain consensus sequence, GXGXXG, as well as two other highly conserved tyrosine kinase consensus sequences, AARN and IHRDL (Hanks et al., 1988). Like other FGF receptors, XFGFR-2 contains a 14-amino acid insertion within the tyrosine kinase catalytic domain (Lee et al., 1989).

Comparison of the XFGFR-2 sequence with other representative members of the FGFR family indicates that XFGFR-2 shares the highest degree of homology (77%) with *bek* (FGFR-2) (Fig. 2). Comparison to other members of the FGFR family indicate that XFGFR-2 shares 64, 62 and 55% identity with *flg* (FGFR-1), FGFR-3 and FGFR-4, respectively (Fig. 2). The highest degree of sequence similarity between XFGFR-2 and other members of the FGFR family is in the highly conserved tyrosine kinase domain. The regions of least similarity are in the extracellular domain, particularly the NH₂-terminal immunoglobulin-like domain and transmembrane domains. Noteworthy is the fact that XFGFR-2 is more similar to FGFR-2 from other species (Fig. 2) than it is to *Xenopus* FGFR-1 (Fig. 1).

XFGFR2	ML—LLALLAFLLVSRITARPSSYSMVDDTTPEPEEPPAKYQISKADVFPVLPGEPLDL	56
XFGFR1	.FSGRS..LWGV.L.GAALSV...PSTLP.EVA.KTK——TEVEPY SAR..DTVT.	
XFGFR2	RCPL-ADGPLVTWTKDGAKLEVNRTLLIVR TYLQIKESTTRDSGLYAC—SVLKNS	109
XFGFR1	Q.R.RE.VQSIS.V.N.VQ.LET...R.TGEEI..SNAGPE.N.....VTIGPSGTYTV.	
XFGFR2	HFFHVNVTEASSSGDDEDDNDGS-EDFTNDNNNIRAPYWTNTEKMEKKLHAVSAANTVKL	168
XFGFR1	FSIN.SDAQP.AED..D..DNS.S.EKASE.SKPNR.F.SHP.....P..K...F	
XFGFR2	RCPARE-PHPSNEWLKNKGEFKQEHRIGGYKVRNQHWSLIMESVVP SDKGIYTCIVENEH	227
XFGFR1	...NGT.S.AL R.....RPDQ.....S.T.....D.....N.....KY	
XFGFR2	GSINHTYHLDVIERSSSHRPILQAGLPANTTAVVGGDAEFVCKVYSDAQPHIRWVRYIEKN	287
XFGFR1	.TL....Q...V...P.....SVT..ST...S.....P....Q.L.H..I.	
XFGFR2	GSRFGVDGLPYFKVLKAAGVNVTDDEEIEVLYVRNVSFEDAGEYTCIAGNSIGISQHS AWL	347
XFGFR1	...VAS..F..VEI..T...TS.KDM...HL...T....Q...L.A.....H.....	
XFGFR2	TVHPAPVNPLEDNPVYMEIGIYSTGIFIFIICMVVVCVCRMRQAKKKKNFTGPPVHK	407
XFGFR1	..LEVEDDKPALLAS.LQL..I..C..AAFVSA...TIIIFK.KHPS..SDFNSQLA...	
XFGFR2	LTKRIPLHRQVTVSADSSSSMNSTTPLVRITRLLNSTDAMPLANVSEYELPHDPMWEFS	467
XFGFR1	.A.S...R.....G..N...H.GVI...PSR-.S.SGTPM.SG.....E..R..VA	
XFGFR2	RDKLTTLGKPLGEGCFGQVMAEALGIDKERPKESVTVAVKMLKDNATEKDLADLVSEMEM	527
XFGFR1	..R.I.....I.L...K.NRVTK..L....SD.N...S..I.....	
XFGFR2	MKMIGKHKNIINLLGACTQGGLTYVIVEYAAKGNLRQYLRARRPLEMEYSFDVTRVPDEQ	587
XFGFR1D.P.....S.....E.....PG...CYNPMCA..QL	
XFGFR2	MTFKDLVSCYQIARGMEYLASQKCIHRDLAARNVLVTENNVKIRDFGLARDVNNIDYY	647
XFGFR1	LS.....A..V.....K.....D.....A.....IHH....	
XFGFR2	KKTSNGRLPVKWM APEALFDRVYTHQSDVVSFGVLMWEIFTLGGSPYPGIPVEELFKLLK	707
XFGFR1	...T.....I.....L.....V.M.....	
XFGFR2	EGHRMDKPANCTNELYMMRDCWHAIPSHRPTFKQLVEDLDRILTLTNEEYLDLSAPLE	767
XFGFR1T.....K.....M..Q....N.....A.SS.Q.....M.VN	
XFGFR2	QYSPSFPDSSCSASSSSGDDSVFSPDMPHPDCLPKFQHVNGVVKT	813
XFGFR1	...C...TR-.STC...E...H..L.DE...YSNG-.LK.R	

Fig. 1. Alignment of the predicted XFGFR-2 amino acid sequence with that of FGFR-1. The sequences were aligned using PC Gene software (Intelligenetics). Gaps in the sequence are positioned with dashes and are introduced to maximize alignment. The dots indicate identities. Amino acids are numbered at the right. The sequence has been entered in the GenBank/EMBL database, accession no. X65943.

Taken together these data suggest that XFGFR-2 is the *Xenopus* homologue of the human and mouse *bek* genes (FGFR-2) and the chicken *cek-3* gene.

Cross-linking of ^{125}I -FGF-2 to XFGFR-2 expressed in COS1 cells

To determine whether XFGFR-2 cDNAs encode functional FGFR, we cloned an XFGFR-2 cDNA into a eukaryotic expression vector and transfected it into COS1 cells. Expression of XFGFR-2 was determined by an ^{125}I -FGF-2 affinity cross-linking assay. COS1 cells transfected with either pcDNAIneo-XFR2.6 or pcDNAIneo alone as a control, were incubated with ^{125}I -FGF-2 in the presence or absence of excess unlabeled FGF-2. After extensive washing to remove unbound ligand, the cells were treated with

the cross-linking reagent DSS, cell lysates prepared and subjected to SDS-PAGE as described (Friesel and Dawid, 1991). COS1 cells transfected with the XFGFR-2 expression vector showed two autoradiographic bands, one of $140 \times 10^3 M_r$ and another of $90\text{--}95 \times 10^3 M_r$ (Fig. 3). Both bands can be competed with excess unlabeled FGF-2 (Fig. 3) or FGF-1 (not shown). Control transfected COS1 cells did not give autoradiographic FGFR bands with the film exposure times needed to detect receptor bands in XFGFR-2 transfected cells. However, upon prolonged film exposures, autoradiographic bands of 130 and $90 \times 10^3 M_r$ were detected and are derived from low levels of endogenous FGFR on COS1 cell membranes (Partanen et al., 1991). These results demonstrate that XFGFR-2 encodes a functional FGF receptor.

	XFGFR-2	XFGFR-1	FGFR-1	FGFR-2	FGFR-3	FGFR-4
SP		21	23	43	18	26
IG1		41	41	59	37	28
IG2		70	70	83	68	62
IG3		68	73	79	70	65
TM		38	52	52	29	19
TK1		79	79	89	85	78
TK2		89	90	97	90	81
CT		61	61	78	58	45
overall identity		62	62	77	62	55

Fig. 2. Schematic structure of a prototype FGFR showing the percentage amino acid identity between different structural domains of XFGFR-2, XFGFR-1, human *flg* (FGFR-1), human *bek* (FGFR-2), human FGFR-3 and human FGFR-4. Each structural domain is identified by the following abbreviations: SP, signal peptide; IG1, IG2 and IG3, immunoglobulin-like domains; TM, transmembrane domain; TK1, tyrosine kinase domain 1; TK2, tyrosine kinase domain 2; and CT, carboxy terminal tail. Overall identities for each receptor type are indicated at the bottom of each column.

Temporal and spatial localization of XFGFR-2 mRNA during development

Expression of XFGFR-2 mRNA was examined by RNA gel blot analysis using RNA samples isolated from various stages of *Xenopus* development (Fig. 4A). Expression of an XFGFR-2 transcript of ~4.2 kb did not become significant until after stage 11-12. XFGFR-2 mRNA expression levels peaked around stage 15 and then remained constant through tadpole stages. A low level of maternally derived XFGFR-2 transcripts was detected in egg and blastula stage embryos

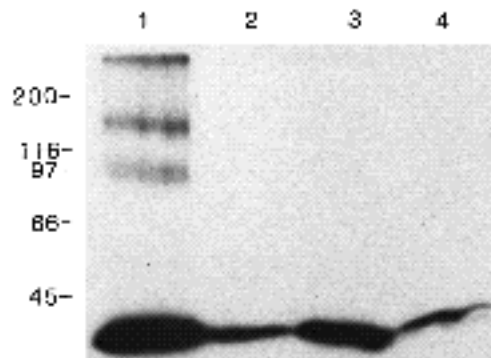


Fig. 3. Cross-linking of ¹²⁵I-FGF-2 to COS1 cells expressing XFGFR-2. COS1 cells transfected with pcDNAneo-XFR2.6 (lanes 1 and 2), or with control pcDNAneo (lanes 3 and 4) were incubated with ¹²⁵I-FGF-2 in the presence (lanes 2 and 4) or absence (lanes 1 and 3) of excess unlabeled FGF-2 at 4°C for 1 hour. Cells were washed and exposed to 0.3 mM DSS for 15 minutes at 4°C. Samples were prepared as described in Materials and Methods and subjected to 9% SDS-PAGE and autoradiography. The migration of the molecular weight standards is shown on the left.

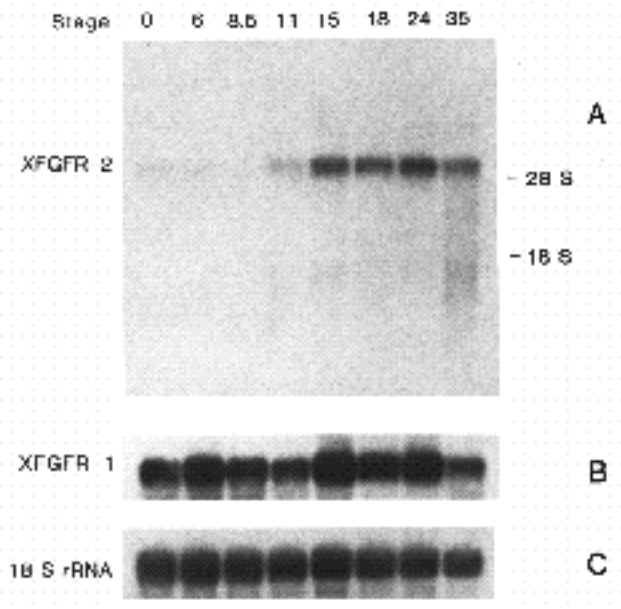


Fig. 4. RNA blot analysis of XFGFR-2 mRNA expression in early *Xenopus* development. Blots containing 10 µg total RNA from eggs and staged embryos were hybridized with probes to XFGFR-2 (A), XFGFR-1 (B) or 18S rRNA (C). Embryonic stages are indicated at the top. The filter hybridized to XFGFR-2 was exposed to film for 3 days, XFGFR-1 for 24 hours, and 18S rRNA for 15 minutes.

upon prolonged autoradiographic exposure of the blots. This is in contrast to the expression of XFGFR-1 transcripts which are expressed at a relatively constant level throughout early *Xenopus* development (Fig. 4B). Rehybridization of these blots to a *Xenopus* 18S rRNA probe demonstrated that equivalent amounts of total RNA were loaded for each stage of development (Fig. 4C).

A detailed analysis of the spatial distribution of XFGFR-2 mRNA was performed by in situ hybridization to whole embryos with digoxigenin-labeled RNA probes (Fig. 5). Expression of XFGFR-2 mRNA can be first visualized at about stage 13-14 (Fig. 5A). At this stage, expression is seen in the anterior neural plate and lateral anterior regions where the presumptive eye anlagen are located. By stage 18 (Fig. 5B), intense hybridization is seen in the dorsoanterior region of the embryo. No hybridization is evident in the ventral or posterior regions of the embryo. At stage 20 (Fig. 5C), expression has resolved into two discrete areas of expression corresponding to regions of the forebrain and the midbrain-hindbrain boundary. At this stage, intense expression continues in the eye anlagen. By stages 26-27 (Fig. 5D), the most prominent region of expression is a sharp band of expression near the midbrain-hindbrain boundary. The area of expression seen in the forebrain has decreased in intensity and may reflect a temporal decrease in expression in this region. At this stage, expression continues to be seen in the eye as well as new expression in the otic vesicle. This expression pattern persists up to at least stage 35. In addition, at larval stages, weak XFGFR-2 mRNA expression is seen in the pharyngeal arches (not shown). Similar hybridization analysis with sense strand RNAs were negative at all embryonic stages.

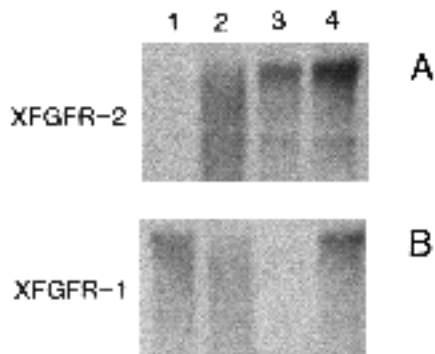


Fig. 6. Expression of XFGFR-2 transcripts in animal caps. (A) Animal pole ectoderm was dissected from stage 8 embryos (10 caps per time point) and incubated for 2 (lane 1), 6 (lane 2), and 24 hours (lane 3) in control medium, or in undiluted XTC-MIF for 24 hours (lane 4). RNAs were isolated, subjected to RNA gel blot analysis and probed with an XFGFR-2 probe. (B) The blot was stripped and reprobed with an XFGFR-1 probe to show differential expression of the two transcripts.

Regulation of XFGFR-2 mRNA expression in animal caps

The observation that XFGFR-2 mRNA expression does not become significant until after the onset of gastrulation indicates that its expression is regulated differently than that of XFGFR-1 mRNA. It has been previously shown by us (Friesel and Dawid, 1991) and others (Musci et al., 1990) that XFGFR-1 expression is maintained in animal pole explants when they are incubated in the presence of a mesoderm-inducing signal, but this expression declines to undetectable levels by control stage 20 when incubated in the absence of mesoderm inducing factors (FGF-1, FGF-2 or activin A).

We examined the expression of XFGFR-2 RNA in explanted animal caps. Animal caps dissected from stage 8 embryos and incubated *in vitro* for 2 hours do not express detectable levels of XFGFR-2 mRNA consistent with the temporal expression pattern observed in intact embryos (Fig. 6A, lane 1). When animal caps were incubated in control medium, XFGFR-2 mRNA expression can first be detected 6 hours post-explantation (Fig. 6A, lane 2) and continues 24 hours after dissection (Fig. 6A, lane 3). This is in contrast to the expression of XFGFR-1 mRNA, for which expression is detectable at the time of explantation (Fig. 6B, lane 1), but not after 24 hours of incubation (Fig. 6B, lane 3) (Friesel and Dawid, 1991; Musci et al., 1990). Incubation of animal caps with XTC-mesoderm-inducing factor (XTC-MIF) resulted in a slight enhancement of XFGFR-2 mRNA expression in animal pole explants after 24 hours of incubation (Fig. 6A, lane 4). Replacement of XTC-MIF with recombinant FGF-1 or FGF-2 gave similar results (not shown). The blots were stripped and rehybridized to an XFGFR-1 probe to demonstrate the dependence of XFGFR-1 expression on the presence of a mesoderm-inducing factor as previously reported (Fig. 6B, lane 4) (Friesel and Dawid, 1991; Musci et al., 1990).

Discussion

We have described the cDNA cloning and developmentally

regulated expression of a member of the *Xenopus* FGFR family. Because of the very high amino acid sequence similarity with the *bek* gene (FGFR-2), particularly in the tyrosine kinase domain, we believe the cloned cDNAs represent the *Xenopus* homologue of *bek* mRNA. In addition to the high degree of amino acid sequence identity, there are several additional features that indicate XFGFR-2 is the *bek* homologue. First, XFGFR-2 has an extracellular domain consisting of three immunoglobulin-like domains. Second, XFGFR-2 has a cluster of five acidic amino acid residues between the first and second immunoglobulin-like domains. This cluster of acidic amino acids is the same in size and position as in FGFR-2 from other species but is smaller than the eight acidic residues found in all of the FGFR-1 genes examined thus far (Dionne et al., 1990; Friesel and Dawid, 1991; Johnson et al., 1990; Reid et al., 1990). Third, the fourteen amino acid insertion in the tyrosine kinase catalytic domain of XFGFR-2 is more homologous to the insert domains of FGFR-2 genes of other species than it is to the insert domain of other FGFR family members.

Alternative splicing of FGFR-1 and FGFR-2 pre-mRNAs has been shown to give rise to multiple variant forms of FGFRs. Indeed, we have shown that at least two splice variants exist for XFGFR-1 (Friesel and Dawid, 1991). The results of RNA gel blot analyses indicate that the major XFGFR-2 transcript is a 4.2 kb species. However, we cannot rule out the possibility that less abundant alternatively spliced forms exist. Given that FGFR-2 from other species gives rise to multiple spliced variants, it is likely that multiple isoforms of XFGFR-2 also exist.

When a full-length XFGFR-2 cDNA was transiently transfected into COS1 cells, new FGF binding sites were expressed at the cell surface. Cross-linking experiments demonstrate that these new FGF binding sites correspond to cross-linked bands of approximately $140 \times 10^6 M_r$ and $90-95 \times 10^6 M_r$. The intensity of the more diffuse lower molecular weight species varied considerably from one experiment to another. The $90-95 \times 10^6 M_r$ species may represent degradation products of the larger $140 \times 10^6 M_r$ species, or alternatively may represent incompletely glycosylated forms of the receptor. Excess unlabeled FGF-1 and FGF-2 abolished cross-linking to both the $140 \times 10^6 M_r$ and $90-95 \times 10^6 M_r$ bands on COS1 cells. These data demonstrate that XFGFR-2 encodes a functional FGF receptor. Like their mammalian counterparts (Dionne et al., 1990), XFGFR-1 and XFGFR-2 bind both FGF-1 and FGF-2. Whether XFGFR-1 and XFGFR-2 bind other members of the FGF family of growth factors is unknown at present. Noteworthy is the recent identification and cloning of two other members of the FGF family from *Xenopus*. One, XeFGF is highly homologous to both FGF-4 and FGF-6 (Isaacs et al., 1992); the other is the *Xenopus* homologue of *int-2* (FGF-3) (Tannahill et al., 1992). Both of these FGFs contain signal peptides and are thus good candidates for inducing factors and may play a role in the formation of the anteroposterior axis. Whether these FGFs are ligands for XFGFR-2 remains to be determined.

The temporal pattern of XFGFR-2 mRNA expression during early *Xenopus* development is different than that of XFGFR-1 mRNA (Friesel and Dawid, 1991; Musci et al., 1990). Whereas XFGFR-1 mRNA expression is relatively

constant throughout early *Xenopus* development, XFGFR-2 transcripts do not accumulate to significant levels until after the onset of gastrulation. Therefore it is unlikely that XFGFR-2 plays a role in the transmission of mesoderm-inducing signals.

During early neurula stages, XFGFR-2 transcripts are detected by whole-mount in situ hybridization in the anterior neural plate and the region of the eye anlagen. In later neurula stages, this expression becomes more discrete, forming two prominent bands of expression, one in the forebrain and the other in the midbrain-hindbrain boundary. Still later, during tailbud stages, expression is detected in the eye and otic vesicle. This distinct pattern of localization suggests multiple roles for XFGFR-2 in patterning of the developing central nervous system in *Xenopus*. This pattern of expression is very similar to FGF-3 (*int-2*) in both the mouse (Wilkinson et al., 1989) and in *Xenopus* (Tannahill et al., 1992). Of particular interest is the expression of *Xenopus* FGF-3 mRNA during neurula stages in the optic cups and the midbrain-hindbrain boundary. In later stages, FGF-3 expression is also found in the pharyngeal arches and the otic vesicle. Despite these similarities, there are some differences in the overall patterns of expression of *Xenopus* FGF-3 and XFGFR-2. We are unable to detect XFGFR-2 mRNA expression in the blastopore region during gastrulation or in posterior regions of the embryo. These differences may be the result of real differences in expression patterns of these two genes or may reflect differences in sensitivity between whole-mount (XFGFR-2) and standard (*Xenopus* FGF-3) in situ hybridization techniques. The spatial localization of XFGFR-2 to many of the same apparent areas of expression of FGF-3 indicate that this receptor-ligand combination may have some functional specificity in embryogenesis. There is little data available on the localization of other members of the FGF family in *Xenopus*, with the exception of XeFGF, whose pattern of expression would not suggest that it is a cognate ligand for XFGFR-2 (Isaacs et al., 1992).

FGFR-2 expression has also been examined in the mouse (Orr-Urtreger et al., 1991). In the mouse, FGFR-2 expression is restricted primarily to tissues derived either from ectoderm or epithelia, whereas FGFR-1 is restricted mainly to the mesenchyme. Although some differences exist in the pattern of expression of FGFR-2 in *Xenopus* and mouse, the results are largely consistent with the fact that FGFR-2 expression seems to be restricted to cells of ectodermal origin.

Recently, Amaya et al. (1991) demonstrated that expression of a dominant negative mutant of FGFR-1 could disrupt normal posterior development in *Xenopus* embryos. It is hypothesized that this occurs by the formation of non-functional homodimers between a mutant FGFR lacking the tyrosine kinase domain and wild-type receptors, thus blocking the normal signaling mechanism. It is likely that this disruption of FGFR function could extend to XFGFR-2 since it was recently demonstrated that FGFR-1 and FGFR-2 can form heterodimers that are capable of ligand-induced transphosphorylation (Bellot et al., 1991; Ueno et al., 1992). The importance of these observations to normal development requires further analysis.

Xenopus FGFR-2 mRNA expression occurs in explanted

animal caps in the absence of exogenously added inducing factors. The timing of expression in XFGFR-2 mRNA animal caps is coincident with its expression in intact embryos. The timing of this expression is not influenced by the addition of inducing factors to the animal caps, although their presence results in a small increase in XFGFR-2 mRNA expression after 24 hours (Fig. 6, data not shown). Although we cannot rule out cell-cell contact as a prerequisite for XFGFR-2 mRNA expression, it does appear to be independent of known mesoderm-inducing growth factors. The mechanism by which XFGFR-2 mRNA expression occurs in the absence of inducing agents is currently under investigation.

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Fig. 5. Whole-mount in situ hybridization analysis of XFGFR-2 expression in *Xenopus* embryos. (A) In situ hybridization of XFGFR-2 to stage 13-14 embryos. This view from the anterior aspect shows XFGFR-2 expression in the anterior neural plate and eye anlagen. (B) In situ hybridization of XFGFR-2 to stage 18 *Xenopus* neurula. Note the strong hybridization signal in the anterior region of this lateral view of the embryo. (C) Anterior view of a stage 20 neurula embryo. Note the paired-stripe pattern of XFGFR-2 expression. (D) Lateral view of a stage 26-27 embryo. Hybridization is seen in the region of the midbrain-hindbrain boundary, eye and otic vesicles. Note that the band of hybridization near the forebrain has decreased in intensity relative to earlier stages.