Expression and functions of FGF-3 in Xenopus development

AURORA LOMBARDO, HARRY V. ISAACS and JONATHAN M.W. SLACK*

Department of Biology and Biochemistry, University of Bath, Bath, United Kingdom

ABSTRACT We have analyzed the expression pattern of the Xenopus FGF-3 gene during early development and examined its biological activity in three different bioassays using Xenopus embryos. We show that from the early gastrula stage there is a domain of expression around the blastopore which becomes a posterior domain as the blastopore closes. An anterior ectodermal domain becomes detectable from mid-gastrula stages in the prospective hind-brain, and there are several later domains of expression: the midbrain-hindbrain junction, the otocyst, the pharyngeal pouches and the tailbud region. By using double whole-mount in situ hybridizations we show that the XFGF-3 expression in the brain is dynamically regulated both in time and space during development. The anterior domain of early neurula stage embryos corresponds to the prospective rhombomeres 3-5. By the time the neural tube is closed, XFGF-3 expression is restricted to r4 and a later new domain of expression is established at the midbrain/hindbrain junction. In addition, we show that, despite its difference in receptor specificity, XFGF-3 can induce the formation of mesoderm from animal caps similarly to other FGFs. It also displays a posteriorizing activity on whole embryos similar to other FGFs. Although the absence of maternal expression makes it unlikely that XFGF-3 is involved in mesoderm induction in vivo, its posterior domain of expression during gastrulation and its posteriorizing activity suggests that it participates in the maintenance of mesodermal gene expression and in the FGF mediated patterning of the anteroposterior axis during gastrulation.

KEY WORDS: Xenopus, FGF-3, FGF receptor, mesoderm induction, posteriorization, rhombomeres, midbrain-hindbrain junction (isthmus)

Introduction

In recent years it has become clear that members of the fibroblast growth factor (FGF) family play several important roles in pattern formation during vertebrate development. During the very early stages of development, FGFs have been shown to be required in Xenopus for the establishment and patterning of the mesoderm (Kimelman and Kirschner 1987; Slack et al., 1987; Amaya et al., 1991, 1993) and for the anteroposterior patterning of trunk and tail (Isaacs et al., 1994; Cox and Hemmati-Brivanlou, 1995; Lamb and Harland, 1995; Pownall et al., 1996). At later stages, work in chicken and mouse has shown that FGFs are involved in a wide range of developmental processes such as the formation of the midbrain (Crossley et al., 1996a), inner ear development (Mansour et al., 1993), the induction and outgrowth of the limbs (Cohn et al., 1995; Crossley et al., 1996b; Vogel et al., 1996; Ohuchi et al., 1997), apoptosis in the tooth (Vaathokari et al., 1996), lung growth (Peters et al., 1994), lens development (Robinson et al., 1995) and the regulation of hair growth (Hebert et al., 1994). Signaling by the members of the FGF family depends upon complex interactions with high and low affinity cell surface receptors that eventually lead to the activation of the MAP kinase signal transduction pathway (Dionne et al., 1991; Johnson and Williams, 1993). Four high affinity receptor genes (FGFR-1 to FGFR-4), members of the tyrosine kinase receptor family, are known to exist. Alternative splicing of the four genes generate multiple variants of the receptors characterized by distinct ligand binding specificities (Johnson and Williams, 1993). Of these the most significant is the distinction between the c type isoforms that bind tightly to FGF-2 and the b type isoforms that bind tightly to FGF-7.

One member of the FGF family, FGF-3 (previously known as int-2), was first identified as a proto-oncogene because of its transcriptional activation in tumors induced by mouse mammary...
tumor virus (Peters et al., 1983; Dickson et al., 1984). Although hardly detected in adult tissues, both mouse and chicken FGF-3 transcripts exhibit a complex pattern of expression in the developing embryo suggesting multiple functions for this growth factor during embryogenesis (Wilkinson et al., 1988, 1989; Mahmood et al., 1995). The knockout of murine FGF-3 gene function (Mansour et al., 1993) causes abnormalities in the tail and defects in the development of the inner ear. Xenopus FGF-3 binds avidly to the FGF2b isoform (Mathieu et al., 1995) and in this regard differs from FGF-2, the factor on which most work has so far been done. Because of this different receptor specificity we felt that FGF-3 might show biological activities distinct from those of FGF-2. In this paper we present a detailed expression pattern of Xenopus FGF-3 during early development and examine its biological activities in three different bioassays, animal cap autoinduction, posteriorization, and bead implantation.

Results

XFGF-3 expression in the Xenopus embryo

XFGF-3 is not expressed maternally but only zygotically, being first detected at the early gastrula stages. At stage 10.5 faint staining is detected in a complete ring around the blastopore with a higher level of expression dorsally (Fig. 1A). As gastrulation proceeds, XFGF-3 expression becomes downregulated on the dorsal side relative to the lateral and ventral aspect of the blastopore (Fig. 1B). At this stage its expression is complementary to that of eFGF, which becomes concentrated on the dorsal side (Isaacs et al., 1995).

In the midline gastrula stage, a prominent stripe of XFGF-3 expression becomes established on each side of the neural plate about half way along the embryo and is maintained throughout neurulation (Fig. 1B-F). As the blastopore closes, the periblastoporal ring becomes concentrated in the posterior (Fig. 1C-E). The level of expression remains reduced in the dorsal midline of the posterior although some faint staining extending up the midline to the anterior stripe is detectable (arrowhead in C and D). By the end of neurulation (stage 20) the anterior stripe has become much narrower and due to the closure of the neural tube has converged towards the dorsal midline (Fig. 1F). Posteriorly, there is still a strong expression in the regions lateral to the closed blastopore that appears to extend for some distance along the developing axis (Fig. 1G).

By the tailbud stages of development XFGF-3 expression has become quite complex. Figure 1H shows that high levels of expression are detectable in the lining of the pharyngeal pouches and in the otocyst. By this stage the expression in the hindbrain has disappeared while a new domain has appeared at the level of the isthmus (midbrain/hindbrain junction). In the posterior, XFGF-3 is expressed in the developing tailbud both in the chordoneural hinge and in the posterior wall of the neuroenteric canal (Fig. 1I).

XFGF-3 expression in the developing Xenopus hindbrain and midbrain

In an attempt to define in more detail to what region of the neuroectoderm the anterior stripe of XFGF-3 expression corresponds, we carried out double whole-mount in situ hybridizations comparing XFGF-3 to three other genes known to be expressed in specific regions of the brain during development: En-2 which is expressed at the midbrain/hindbrain junction (isthmus) (Hemmati-Brivanlou and Harland, 1989), Krox-20 whose two medial stripes of expression correspond to rhombomere 3 and 5 (r3 and r5) of the developing hindbrain (Bradley et al., 1992) and HoxB1, expressed in rhombomere 4 (Godsave et al., 1994).

Throughout neurulation the band of XFGF-3 expression (blue staining) lies posterior to that of En-2 (orange staining) showing that XFGF-3 expression at this stage is restricted to the future hindbrain rather than the isthmus (Fig. 1N). Krox 20 starts to be detectable in the hindbrain, in early neurula stage embryos (st. 13-14), when its expression is initially restricted to the prospective rhombomeres 3 (Fig. 1J, orange staining). At this stage the XFGF-3 domain shares the same anterior boundary but extends about 2 rhombomeres further out to the posterior (Fig. 1J). In slightly older embryos (st. 15), in which Krox 20 is expressed as two bands in rhombomeres 3 and 5, it becomes apparent that XFGF-3 is expressed in r3, r4 and r5 (Fig. 1K). A comparison with Hox B1, which identifies the prospective rhombomere 4 (Fig. 1M), shows that during the neural fold stages (st. 16-17) XFGF-3 is downregulated in the prospective rhombomere 3 but it is still expressed at high levels in the prospective r4 and r5. By neural tube closure XFGF-3 domain has narrowed further and its level of expression has been reduced: at this stage it is absent from r3 and r5 but still present in r4 (Fig. 1L).

By tailbud stages, XFGF-3 expression in the hindbrain has dropped below detection. But a new domain of XFGF-3 expression has arisen at the midbrain/hindbrain junction, shown by the fact that En-2 and XFGF-3 domains of expression are now found to overlap (Fig. 1O).

Effects of FGF-3 overexpression

Mesoderm induction

Direct binding and binding competition analysis have shown that XFGF-3 has a different receptor specificity from the other FGFs in that it binds with high affinity to both b and c splice variants of FGFR-2 while it has only a weak affinity for FGFR-1 (Mathieu et al., 1995). This difference in receptor preference raises the possibility that XFGF-3 might show different biological activities to other members of the FGF family.

In order to test this possibility we have examined the biological properties of XFGF-3 in early amphibian development by injecting various amounts of its mRNA into two cell stage embryos. To assay for mesoderm induction, animal caps were taken from blastula stage embryos and cultured for three days and then scored for the formation of fluid-filled vesicles as an indication of mesodermal induction (Godsave and Slack, 1988; Isaacs et al., 1984) (Table I and Fig. 2).

The histological analysis of the induced animal cap explants shows the presence of muscle tissue, mesenchyme, and mesothelial cells, demonstrating that, in common with other members of the FGF family, XFGF-3 can elicit the formation of ventrolateral type of mesoderm (Fig. 2D). In addition, occasionally (6/76), proctodeum-like structures were seen to be formed in animal caps induced with variable doses (14-56 pg) of FGF-3 mRNA (Fig. 2E-F). The dose-response data presented in Table I show that XFGF-3 has a comparable specific activity as a mesoderm-inducing agent to XFGF-2, XFGF-8 and XFGF-9, but a lower activity by about 10x, than eFGF (Isaacs et al., 1992; Song and Slack, 1996; Christen and Slack, 1997).
Fig. 1. XFGF-3 expression in the developing Xenopus embryo. (A) Vegetal view of an early gastrula stage embryo (st.10.5); dorsal to the top. A faint staining is detected in a complete ring around the blastopore with higher levels of expression visible dorsally. dl, dorsal blastopore lip. (B) Dorsoventral view of a late gastrula stage embryo (st.12.5) showing the periblastoporal expression of XFGF-3. Higher levels of XFGF-3 mRNA are present in the lateral and ventral aspects of the blastopore. Arrowheads point to the anterior stripe of expression that begins to be visible at this stage on each side of the neural plate. yp, yolk plug. (C-E) Early neurula stage embryos. (C) Dorsal views of a st13 and st14 embryos, respectively (anterior to the left). Expression in the neural plate has become more prominent by this stage. A faint staining is detected either side of the midline extending up to the anterior stripe (black arrowheads in C and D). (D) Posterior view of a st.13 embryo showing XFGF-3 expression around the closed blastopore (bp). (F) Dorsal view of a cleared embryo at the end of neurulation stage (st.20). The anterior stripe of XFGF-3 expression has become much narrower and has converged towards the midline. (G) Posterior view of a stage 20 embryo showing a strong expression in region lateral to the closed blastopore (bp) extending for some distance along the developing axis. (H-I) Tailbud stage embryo (st.32). (H) Side view of the head region of a cleared embryo showing expression in the branchial arches (bra), in the otic vesicle (otv) and at the level of the midbrain/hindbrain junction (mjh). (I) Side view of the posterior region of a cleared embryo showing XFGF-3 expression in the tailbud in the chordoneural hinge (cnh) and in the posterior wall (pw) of the neurenteric canal. (J-L) Double whole-mount in situ hybridizations showing XFGF-3 and Krox 20 expression (blue and orange staining, respectively). (J) Early neurula stage embryo (st.14). Krox 20 and XFGF-3 expression are found to overlap at the level of the prospective rhombomere 3 of the hindbrain. At this stage Krox 20 expression in the prospective rhombomere 3 is only barely detectable (arrowhead). (K) Stage 15 embryo. XFGF-3 at this stage is expressed in rhombomeres 3-5. (L) Stage 20 embryo. By the end of neurulation the domain of XFGF-3 expression has narrowed, being restricted to the prospective rhombomeres 4. (M) Double whole-mount in situ hybridization showing XFGF-3 and Hox B1 (orange staining) expression in a stage 17 embryo. Hox B1 band of expression identifies the prospective rhombomere 4. (N) Double whole-mount in situ hybridization showing En-2 (orange staining) and XGF-3 expression. (O) Neurula stage embryo (st.16). Throughout neurulation the band of XFGF-3 expression lies posterior to that of En-2. (P) Side view of a tailbud stage embryo (st.30). By tailbud stages XFGF-3 expression in the hindbrain has disappeared while a new domain of expression has arisen at the isthmus (arrowhead) that overlaps with that of En-2.

In order to test whether XFGF-3 function is subject to the inhibitory effect of the dominant negative FGFR-1 receptor (FGFR-1: Amaya et al., 1991), we co-injected XFGF-3 mRNA with an excess of XFD mRNA. Table 1 shows that XFD can effectively block XFGF-3 inducing activity indicating that the receptors stimulated by XFGF-3 can be inhibited by this reagent.

Posteriorization

In whole embryos, overexpression of eFGF during gastrula stage causes a reduction of anterior structures (Isaacs et al., 1994; Pownall et al., 1996). This effect can also be observed following injection of mRNA into fertilized eggs. We found that XFGF-3 mRNA would cause anterior truncation in a dose-dependent way. Low doses (up to 35 pg RNA) cause mild to moderate anterior truncations characterized by a partial or complete loss of the eyes and the cement gland (Fig. 2G). Higher doses (70 to 280 pg) lead to a severely posteriorized phenotype characterized by a loss of most of the head (Fig. 2H). The trunk is abnormally bent ventrally and an enlarged proctodeum region is found at the posterior of some embryos whereas the tail seems only lightly affected. Although these results differ somewhat from those seen with eFGF mRNA (Isaacs et al., 1994) in that XFGF-
overexpression does not significantly affect normal gastrulation, they are comparable to those seen with FGF-2 (Thompson and Slack, 1992), FGF-8 (Christen and Slack, 1997) and FGF-9 (Song and Slack, 1996). Again, the results of our analysis have shown that although XFGF-3 has a different receptor preference from other members of the family, it nonetheless appears to share their biological activities.

Later effects
Because of the multiple late expression domains we considered it possible that XFGF-3 would show novel biological activities associated with these domains. To investigate this we grafted heparin beads soaked in XFGF-3 protein (370 μg/ml) into various sites in the neurula.

XFGF-3 beads implanted anteriorly between early neural fold and early tailbud stages (st. 15 to st. 22) were found to affect the differentiation of the pigmented epithelium of the eye and to cause abnormalities in the layer structure of the retina in 15 out of 20 embryos (Fig. 2J), while posterior bead implantations at early neural plate stage (st. 13-14) were found to induce the formation of ectopic otic vesicles in 2 out of 4 embryos (Fig. 2K, L). However, the effects found with XFGF-3 were very similar to those elicited by the implantation of FGF-2 beads in similar stage embryos (Lombardo and Slack, 1998) and no specific effects that are not shown by FGF-2 were found in our study. Moreover the indications are that the results with similar doses of FGF-2 are stronger than with XFGF-3.

Discussion
In this study we have extended our characterisation of the Xenopus FGF-3 expression pattern published in an earlier report (Tannahill et al., 1992), and also examined its biological activity in various assays using Xenopus embryos.

By using double whole-mount in situ hybridizations we have been able to show that XFGF-3 expression in the brain is complex, being dynamically regulated both in time and in space during development. These results are comparable to those reported for both the chicken and mouse homologs (Mahmood et al., 1995). However, the pattern revealed by our study does differ slightly from what other investigators have observed. Expression was reported in r5 and r6 in both the chicken and the mouse hindbrain (Wilkinson et al., 1988; Mahmood et al., 1995), whereas we have not seen expression in r6 in Xenopus. In addition, although r4
appears to be a common site of FGF-3 expression in mouse, chicken and Xenopus, neither in mouse nor in chicken has expression been detected in r3 (Wilkinson et al., 1988; Mahmood et al., 1995). Mahmood et al. (1995) do detect FGF-3 expression in the unsegmented hindbrain of the chick, although they do not comment on what prospective rhombomere/s this region may correspond to. Thus, it remains possible that the early domain of expression that Mahmood et al. (1995) describe in the unsegmented chicken hindbrain does encompass the prospective r3 and that, due to the highly dynamic pattern of expression of this gene, FGF-3 expression at this level is lost by the onset of rhombomere formation.

As far as biological activity is concerned, we have shown that, despite its difference in receptor specificity, XFGF-3 can induce the formation of mesoderm from animal caps similar to other FGFs. It also displays a posteriorizing activity on whole embryos similar to other FGFs. The absence of maternal expression makes it unlikely that XFGF-3 is involved in mesoderm induction in vivo, but its posterior domain of expression during gastrulation and its posteriorizing activity indicate that it may participate along with eFGF and FGF-8 in the maintenance of mesodermal gene expression and in the FGF mediated patterning of the anteroposterior axis during gastrulation. The biological activities shown in the bead implantation experiments, together with the expression data, also make it likely that FGF-3 contributes to the inductive activity of the isthmus region in the brain, as well as being necessary for the correct morphogenesis of the ears.

The inducing activity of XFGF-3 is completely inhibited by the dominant negative receptor XFD (Amaya et al., 1991). This is a truncated form of FGR1, but its ability to inhibit XFGF-3 suggests that it can block other receptors in addition to R1 itself. A detailed study of FGF receptor expression in Xenopus has yet to be carried out, but in the Spanish salamander, Pleurodeles waltli, the presence of several types and isoforms of FGF receptors was reported (Shi et al., 1992; 1994; Launay et al., 1994; Riou et al., 1996). Our results show that the Xenopus blastula must contain receptors that are stimulated by FGF-3, and also that such receptors can be blocked by XFD. Therefore some of the function of endogenous FGFs, deduced from the phenotype of the XFD embryos (Amaya et al., 1991; 1993; Isacs et al., 1994) is likely to be attributable to XFGF-3 signaling. In summary, our results suggest that XFGF-3 contributes to the total endogenous FGF activity, but that it does not have any unique activity of its own.

Materials and Methods

Embryological methods and RNA injections

Embryos were cultured according to the methods of Godsave and Slack, 1988 and staged according to Nieuwkoop and Faber, 1967.

To create the CS2-FGF-3 plasmid a 5' primer, GAGAGAGATCGATAC-ATATGGTTATAATTTGGATCCT and a 3' primer, CTGATCCTGCAGACTAGTCAATGGCTCTCCTGTTTGTCT containing a Pst I restriction site (underlined), were used to amplify a 736 bp fragment containing the full Xenopus FGF-3 coding region from the plasmid XFGF3.1 (Kiefer et al., 1993). The amplified PCR product was then cut with Cla I and Pst I restriction enzymes and ligated between the corresponding sites in the CS2 vector (Turner and Weintraub, 1994).

FGF-3 synthetic mRNA was produced by in vitro transcription using the MEGAscript SP6 kit (Ambion), according to the manufacturer’s instructions, using as template CS2-FGF-3 plasmid linearized with Not I. Injections were performed as described in Isacs et al., 1994. Different amount of synthetic mRNA dissolved in 10 ul of water were injected into each blastomere of two cell stage embryos in NAM+5% Ficoll. At stage 6 the embryos were transferred to NAM+10% Ficoll and allowed to develop until the appropriate developmental stage. Animal cap explants were taken at stages 8-9 and cultured in NAM/2 until the appropriate control stage was reached, at which time they were fixed for histological analysis.

Histological methods were as described in Godsave and Slack, 1988.

Preparation of XFGF-3 protein and bead implants

XFGF-3 protein was purified from the conditioned medium from COS-1 cells transfected with the plasmid XFGF3.1 as described in Mathieu et al. (1995) and bead implantations were performed as described in Lombardo and Slack (1998). The concentration of FGF-3 protein used in this study was 370 µg/ml. For control experiments, beads were incubated in PBS instead of XFGF-3.

In situ hybridization

Albino embryos were cultured to various developmental stages and then fixed in MEMFA (0.1 M MOPS, 2 mM EDTA, 1 mM MgSO₄, 3.7% formaldehyde) for an hour at room temperature and stored in 100% ethanol at -20°C until further processing.

Whole-mount in situ hybridizations were carried out as in Harland (1991), with modifications as in Pownall et al., 1996.

For double in situ hybridizations the probes were transcribed from linearized plasmids using either 10X DIG RNA labeling mix (FGF-3) or 10X Fluorescein RNA labeling mix (Boehringer Mannheim) (En-2, Krox 20 and HoxB1).

FGF-3 was linearized with Bam HI and transcribed with T3 polymerase. En-2 and Krox 20 were detected as in (Doniach et al., 1992). HoxB1 (Godsave et al., 1994) was linearized with Ndel and transcribed with SP6 polymerase.

Hybridization of probes to endogenous RNAs was detected by anti-DIG (XFGF-3) or anti-fluorescein (En-2, Krox-20, HoxB1) alkaline phosphatase conjugated antibodies. XFGF-3 expression was visualized by a color reaction using BM-purple (Boehringer Mannheim) (blue staining) while to visualize that of En-2, Krox 20 and HoxB1 INT/BCIP (Boehringer Mannheim) was used (red/orange staining).

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References


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