Inductive effects of fibroblast growth factor and lithium ion on *Xenopus* blastula ectoderm

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**Summary**

We have studied the response of *Xenopus* blastula ectoderm to fibroblast growth factor and to lithium ion. The properties of acidic and basic FGF are very similar showing a 50% induction level at 1–2 ng ml\(^{-1}\) and a progressive increase of muscle formation up to concentrations of 100–200 ng ml\(^{-1}\). The elongation of explants also shows a dose–response relationship. The minimum contact requirement for induction of ectoderm explants is about 90 min and the stage range of ectodermal competence extends from midblastula to early gastrula, both these figures resembling those obtained in embryological experiments with vegetal tissue as the inducer. Lithium chloride concentrations which produce anteriorization of whole embryos have no effect on isolated ectoderms unless accompanied by FGF. Simultaneous treatment with FGF and Li lead to a marked enhancement of both elongation and muscle formation over that produced by FGF alone. By contrast, ventral marginal explants show increased elongation and muscle formation if treated with lithium alone suggesting that they have already received a low-dose FGF treatment within the embryo. It is concluded that endogenous FGF may be solely responsible for inducing the ventral mesoderm and that dorsalization of ventral mesoderm to the level of somitic muscle might be achieved either by a very high local concentration of FGF in the dorsal region, or by the action of a second, synergistic, agent in the dorsal region.

Key words: heparin-binding growth factors, fibroblast growth factors, lithium ion, mesoderm induction, muscle induction, organizer, dorsalization.

**Introduction**

The nature of the morphogens that control the spatial pattern of specification in the early embryo has long been a mystery. Interest in this problem was recently stimulated by the discovery of a *Xenopus* cell line which secretes a mesoderm-inducing factor (Smith, 1987), and shortly afterwards we reported that a small group of pure heparin-binding growth factors (HBGFs) were active as mesoderm-inducing agents when tested on isolated ectoderm from *Xenopus* blastulae (Slack *et al.* 1987). They were basic fibroblast growth factor (bFGF), acidic fibroblast growth factor (aFGF) and embryonal carcinoma derived growth factor (ECDGF). More recently, Kimelman & Kirschner (1987) have obtained evidence for the presence of an mRNA of the bFGF type in *Xenopus* embryos. Together these results suggest that FGF may be a morphogen responsible for inducing mesoderm during early *Xenopus* development, but in order to assess the possible role of the endogenous FGF we need an accurate knowledge of the response of competent cells. In the present paper, we have made further studies with both a and bFGF, which give essentially identical results, and examined the following features: (i) the amount of muscle formed at different concentrations, measured both histologically and by Western blotting, (ii) the elongation of the explants that occurs while control embryos are gastrulating, (iii) the minimum contact time required for induction and (iv) the range of stages over which the ectoderm remains competent. The results are all consistent with a role for endogenous FGF as an inducer of ventral-type mesoderm *in vivo*.

However, one morphogen is not enough to account for the formation of pattern over a 2- or 3-dimensional embryo. A substance emitted from a source of any shape and spreading by diffusion would be expected to induce concentric rings of tissue to
similar states of specification, the cell state mapping one-to-one onto the concentration gradient. The specification map of the early *Xenopus* mesoderm is not like this and shows a localized region of extreme dorsal character called the organizer and an extended ring of extreme ventral character (later forming mesenchyme and blood cells). The main intermediate mesodermal-derived tissue, the skeletal muscle of the myotomes, is, according to our results, induced later by a secondary interaction between these two initial mesodermal primordia (Slack & Forman, 1980; Dale & Slack, 1987b).

In the present paper, we show that LiCl, an agent which perturbs several second message pathways and has a striking anteriorizing effect on whole embryos (Kao *et al.* 1976; Cooke & Smith, 1988), has an activity complementary to the HBGFs in that it will dorsalize ventral mesoderm but not mesodermalize ectoderm. Application of the two agents together to ectoderm explants will produce mesoderm inductions with a high muscle content. Although we do not wish to suggest that lithium is a natural morphogen we feel that these results strengthen the case for the existence of morphogens additional to FGF and that the pattern of territories in the blastula becomes specified in response to a combination of morphogen concentrations.

**Materials and methods**

**FGF**

a and bFGF used for biological experiments were prepared from bovine brain as described by Gospodarowicz *et al.* (1984), Esch *et al.* (1985) and Lobb *et al.* (1986). The steps are: ammonium sulphate precipitation, CM-Sephadex, Heparin Sepharose, and Mono S FPLC. After this the aFGF appeared completely pure by reverse-phase HPLC and by amino acid composition analysis; the bFGF still contained some minor impurities which could be resolved by reverse-phase HPLC. The concentrations were deduced from the amino acid analyses of reverse-phase-purified material and enabled us to determine the specific activities of both forms of FGF as 0.5–1 x 10^6 inducing units mg^-1. For mesoderm-inducing factors generally, we have defined 1 unit ml^-1 as the concentration which is just sufficient to cause induction of an explant, or, if a population of explants is treated, is sufficient to cause induction in 50% of explants (Cooke *et al.* 1987; Godsaver *et al.* 1988). Although some differences between a and bFGF have been reported in their capacity as endothelial cell mitogens (Gospodarowicz & Cheng, 1986), their properties as inducing factors appear identical.

**Other substances**

Dibutyryl cAMP, dibutyryl cGMP, TPA and A23187 were purchased from Sigma.

**Western blots**

These were used for measuring the myosin content of explants induced by FGF. The samples, representing one explant, were separated on a 5% gel (Laemmli, 1970), equilibrated for 30 min in transfer buffer (Towbin *et al.* 1979) containing 0.1% SDS, and transferred to nitrocellulose for 14 h in a Hoefer Transphor apparatus. The nitrocellulose sheets were blocked for 1 h in 0.15 M NaCl, 0.1 M Tris–HCl pH 7.4 containing 1% w/v Milk powder and then stained with a 1:200 dilution of an antibody to *Xenopus* muscle myosin (see Dale *et al.* 1985) in the same buffer for 1 h. The form(s) of myosin recognized by this antibody start to accumulate in somitic muscle from about stage 35 and are abundant by stage 40–41 when the cultures are terminated. The blots were washed 3 x 10 min in the same buffer without milk but with 0.1% Tween 20, then reacted with 1/100 125I-donkey anti-rabbit (Amersham International) in the same buffer followed by several washes, drying and autoradiography. The film was pre-flashed to an optical density of 0.1–0.2 and the bands quantified by scanning with a Gelman DCD-16 gel scanner, or cutting out from the blot and counting in a gamma-counter. The procedure was calibrated by including in each run a set of tracks containing known amounts of *Xenopus* myosin. This corrects for losses during transfer and for non-linearities in antibody binding or autoradiography and allows for quantitative comparison between induced explants and explants of somitic muscle from control embryos. Where protein content of samples was measured this was by the Folin method on dialysed gel samples.

**Embryological methods**

*Xenopus* embryos were obtained by artificial fertilization. Methods for fertilization, composition of salines, histological techniques and inducing factor assays are all given in Godsaver *et al.* (1988). The stage series is that of Nieuwkoo & Faber (1967). Animal pole explants were dissected as described in Dale *et al.* (1985) and ventral marginal zone explants, following vital staining of the dorsal side, as in Dale & Slack (1987b). Embryos were dissected at late stage 7 (256 cells) or early stage 8 (512 cells). 2 h treatments with LiCl or FGF were at room temperature (22°C) and culture at 25°C.

For the measurement of lithium uptake, groups of 20 whole animal hemispheres were exposed for 2 h. They were then rinsed twice in NAM, homogenized in 0.1 ml 10% TCA and microfuged. The Li content of the supernatant was measured by atomic emission spectroscopy using a preparation from unexposed explants as a blank. The volume of tissue was estimated by measurement of total protein in the TCA pellet and compared with the protein content of a whole embryo (volume 144 μl) processed in the same way. The derived intracellular lithium concentration was 6.4 mm. Protein was measured by the Folin method.

**Results**

**Dose–response curve for FGF**

In our previous publication, we presented a dose–
Fig. 1. Dose–response curves for *Xenopus* ectoderms exposed to bovine aFGF. (A) The percentage of explants induced (filled circles) and the average myosin content/explant (filled triangles). Data compiled from 111 explants from 4 egg batches. (B) Lengths of explants after overnight culture (control stage 22). Each point represents a mean for 6 explants with standard error shown.

response curve for bFGF (Slack *et al.* 1987). Here we show results for aFGF (Fig. 1A) but with a much more accurate quantification of the amount of muscle found in the inductions. It is striking that the dose–response relationships of the acidic and basic forms of FGF seem to be essentially identical. The upper curve shows the proportion of explants forming swollen vesicles after 3 days of culture. Histological studies have shown that unswollen explants contain only atypical epidermis while swollen ones always contain some mesoderm, even if it is only a small amount of mesenchyme and mesothelium. There is a very steep rise from 0 % to 100 % with 50 % of explants becoming induced at a concentration of 1–2 ng ml⁻¹, or about 100 pM. The lower curve shows the amount of muscle formed, measured by Western blotting (also see Fig. 3A,D). It may be seen that there is a plateau region from about 2 to 15 ng ml⁻¹ over which most of the explants are induced but the muscle content is low and above this concentration the muscle content rises steeply. Using the same method we have found that the myosin content of pure somitic muscle from 3-day embryos is about 33 % of total protein and, taking the average protein content of an explant to be 7 μg, this enables us to deduce that the average muscle content of the explants treated at 170 ng ml⁻¹ is 24 %. At higher concentrations than this some inhibition of differentiation is found so the maximum muscle content inducible with FGF is probably around this value. We have also measured the muscle content of many specimens histologically by tracing onto graph paper with a camera lucida. The outline of the muscle block and of the whole specimen was traced from every fifth section and the volume of the muscle was expressed as a proportion of the whole specimen. This method gives a maximum muscle content of about 10 %, and since differentiated explants contain much empty space we feel that this is consistent with the 24 % figure from the biochemical measurements. It is known that the pigmented surface of the explants is impermeable to inducing factors (Cooke *et al.* 1987) and so probably quite a high proportion of the exposed cells become committed to form muscle as a result of high-dose exposure to FGF. Whether such doses can be regarded as physiological is another matter which is discussed below. In contrast to the ubiquitous appearance of mesenchyme and mesothelium, and the frequent appearance of muscle, only one case out of several hundred examined histologically has contained any notochord even though doses have ranged as high as 1 μg ml⁻¹. In this regard, FGF seems to differ in its biological effects from the mesoderm-inducing factors of the XTC/WEHI class which induces notochord reproducibly (Smith, 1987; Godsave *et al.* 1988).

Fig. 1B shows the mean lengths of a group of explants exposed to aFGF overnight. Previous work has shown that the elongation somewhat resembles the gastrulation movements of an intact embryo and represent the earliest known marker of mesoderm induction (Symes & Smith, 1987). In the case of FGF, we have found that the elongations commence during gastrulation (from stage 10) and may continue during the neurulation and subsequent elongation of control embryos. The length at a given time always increases with dose, probably reflecting a greater proportion of induced cells within the explant. However, the absolute values of the elongations show considerable variability both between individual
cases and between egg batches and are therefore less useful as quantitative measures of the response than muscle formation.

Ventral marginal zone (VMZ) explants from stage-7 to -8 embryos, which normally self-differentiate into ventral-type mesoderm, were also treated with aFGF (17 ng ml$^{-1}$) but showed no change in shape and no extra muscle formation.

**Competence of the ectoderm**

Our early experiments involving short-term treatments with FGF gave somewhat variable results which were eventually ascribed to absorption of FGF onto the agar bases of the dishes used. When dishes are coated with electrophoretic grade agarose this problem does not arise and the results presented here were obtained in this way. The minimum contact requirement was determined by exposing stage-8 ectoderm explants for different times then rinsing in NAM, culturing and scoring for vesicles. The results are shown in Fig. 2A and it may be seen that the proportion of inductions rises to a plateau after about 90 min. This is very similar to the estimate of 1.5–2.5 h for the minimum contact time using vegetal pole tissue as the inducer (Gurdon et al. 1985), and somewhat longer than the estimate of 10 min from Cooke et al. (1987) using conditioned medium from XTC cells.

To determine the stage of onset of competence, ectoderm explants of different stages were exposed to bovine FGF for 90 min (Fig. 2B). This showed that the response was feeble for the early stages but rose to a high plateau by stage 7 (128 cells). To determine the time of cessation of competence, explants from different stages were exposed to FGF indefinitely, and this showed that the major drop came between stages 9 and 10, around the beginning of gastrulation (Fig. 2C). Once again these results show good agreement with embryological experiments as Jones & Woodland (1987) have recently used heterochronic combinations to estimate the commencement of competence between stages 6 and 7 and its disappearance between stages 10 and 10.5. We have also measured the muscle content of many of these specimens by histological methods and found that the highest values for a given concentration of FGF were found for specimens treated between stage 7 and 9.

**Effects of lithium ion**

Lithium ion has long been known to have interesting effects on the morphology of many embryo types including amphibians (Lehmann, 1937; Masui, 1956) and it was thought desirable to re-examine its effect now that the biochemical basis of early regional specification is being uncovered.

The standard solution of Li was one in which the NaCl of the NAM salts is entirely replaced by LiCl. The Li concentration is thus 0.1 M although sodium ion is still present at 9.2 mM from the sodium phosphate buffer. This solution is toxic in the long term and so treatments were given for 2 h at 22°C which had no ill effects on cell viability or differentiation.

Several batches of intact embryos were treated in this way starting at stages from 32 to 512 cells to see...
whether the 'lithium syndrome' described by Kao et al. (1986) could be reproduced. The results of this were extremely variable from batch to batch. Some showed no effect at all, some showed only a few mild cases while others showed 100% extreme anteriorization (see Fig. 4). This variability was not due to differences in stage at the beginning of treatment and was not shown in explant experiments, suggesting that its cause lies in a variation of ease of penetration of the embryo's outer surface.

Ectoderm explants receiving the standard Li treatment were entirely unaffected and, like controls, formed only epidermis. However Li + FGF together gave rise to extreme elongations and high levels of induced muscle (Figs 3 and 5). In terms of muscle formation, the lithium had an effect equivalent to raising the FGF concentration by a factor of about 10. The lithium enhancement is shown with both a and bFGF. Although simultaneous treatment was most effective, an effect was also found when the Li was given for 2h before or after a 2h treatment with the FGF. The mechanism of Li action is still not really understood and it may affect more than one pathway, however the lack of requirement for simultaneous treatment suggests that the responses to both FGF and Li probably have a time course of an hour or two.
Fig. 5. Elongation of ectoderm explants treated at stage 7 and cultured overnight (control stage 22). (A) Untreated. (B) 0-1 M-LiCl 2 h. (C) aFGF 17 ng ml\(^{-1}\) 2 h, showing slight elongation. (D) aFGF + LiCl 2 h, showing substantial elongation. Bar, 0-4 mm.

rather than a minute or two.

In order to obtain an estimate of the intracellular lithium concentration, 20 isolated animal hemispheres were exposed for 2 h and then their lithium content was measured by atomic emission spectroscopy. This gave a value of 6-4 mM, a little higher than that measured by Beckett et al. (1987) for intact embryos. The intracellular concentration is evidently much lower than the extracellular level of 0-1 M which suggests that even the blastocoelic surface of blastula cells presents a considerable barrier to Li entry, but that the intracellular concentration is probably high enough to affect both inositol lipid and cAMP metabolism (Drummond, 1987).

Although *Xenopus* ectoderm fails to respond to LiCl alone, explants of ventral marginal tissue responded in a spectacular fashion giving extreme elongated structures and later forming almost solid masses of muscle (Figs 3 and 6). It would appear, therefore, that ventral marginal zone has already experienced the equivalent of a low-dose FGF treatment, which is entirely consistent with the typical differentiation patterns of such explants, differing from FGF inductions only in the additional presence of blood cells.

Other factors

In attempts to approach the second message level of mechanism, the following compounds have been tested alone or with aFGF: diBu cAMP and diBu cGMP (3–100 \(\mu\)g ml\(^{-1}\)), A23187 (0-5 \(\mu\)g ml\(^{-1}\)), TPA (100 ng ml\(^{-1}\)), A23187 + TPA, but none have yielded any inductions alone or enhancements of elongation or muscle formation with FGF.

Discussion

The results presented above on the dose–response curves for proportion of induced explants, elongation and muscle formation suggest that the inductive properties of the bovine FGFs resemble those of the ventral and lateral parts of the vegetal hemisphere of the blastula (Dale & Slack, 1987b). Furthermore, the results obtained for minimum contact time, onset of competence and decay of competence in the ectoderm are similar to those obtained in the analogous embryological experiments (Gurdon et al. 1985; Jones & Woodland, 1987). Kimelman & Kirschner (1987) have found a bFGF-like mRNA in eggs and embryos and we ourselves have recently obtained evidence for the presence of an HBGF protein which closely resembles bovine bFGF in its specific activity, biological effects and immunological reactivity (J.M.W.S., unpublished data). These considerations make us feel that it is legitimate to attempt to explain events within the embryo by reference to *in vitro* experiments with the heterologous factors. So, to what extent can we explain the mesoderm induction process by the known properties of the FGFs?

Mechanism of mesoderm induction

On the basis of embryological experiments we have distinguished three component events: induction of the ventral mesoderm, induction of the organizer and
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Fig. 6. Dorsalization of ventral marginal zone explants by treatment with LiCl at stage 7. A,B after overnight culture; C,D after 3-day culture. (A) Control, bar, 0.5 mm. (B) Treated 2h LiCl showing substantial elongation, bar, 0.5 mm. (C) Section of control showing ventral mesoderm, bar, 50 μm. (D) Section of Li-treated VMZ showing massive muscle block, bar, 50 μm.

dorsalization of part of the ventral mesoderm by the organizer (reviewed Smith et al. 1985). A probable role for the endogenous FGF is as the inducer of the ring of ventral-type mesoderm extending over the ventrolateral 270° or more of marginal zone circumference in the blastula. Experiments involving isolated ectoderm have shown that exposure to low doses of purified FGF alone is sufficient to induce mesenchyme and mesothelium although formation of the blood islands may involve a further influence from the endoderm (Maufroid & Capuron, 1985). The fact that ventral marginal zone explants can be dorsalized by lithium alone suggests that they have already experienced the equivalent of a low-dose FGF treatment in the embryo.

On the other hand, it is probable that the endogenous FGF is not responsible for the induction of the organizer. The organizer is defined as that part of the mesoderm that will differentiate partly into notochord and that will dorsalize other mesoderm (Slack, 1983), and it has been shown that it is induced only by endoderm from the most dorsal part of the blastula (Gimlich & Gerhart, 1984; Dale & Slack, 1987b). Since FGF will not reliably induce notochord at any concentration, it is likely that the induction of the organizer is due to something else. In our hands, both LiCl and TGFβ together with FGF have yielded a few notochord-containing inductions but this effect is not reproducible, so if the endogenous FGF is responsible for induction of the organizer it probably does so in conjunction with some other as yet unknown agent. Alternatively the organizer may perhaps be induced by a mesoderm-inducing factor of the XTC/WEHI class (Smith, 1987; Godsave et al. 1988), which now seems likely to be another molecule of the TGFβ family called TGFβ-2 (Rosa et al. 1988). It has been shown that an organizer can be induced by XTC-MIF (Cooke et al. 1987) although there is as yet no evidence for the presence of such factors in the embryo.

The third process that we have defined by embryological experiments is the dorsalization of the ventral mesoderm by the organizer to form striated muscle and pronephric tubules. Could endogenous FGF also be responsible for this? Because of the wealth of molecular and immunological markers that have been available (Dale et al. 1985; Gurdon et al. 1985; Jones & Woodland, 1987) the discussion is most easily conducted in terms of striated muscle. All of the muscle arising up to the tailbud stage in an amphibian embryo comes from the somites (Mohun et al. 1980, 1984). Fate-mapping experiments have shown that most of the somites derive, in normal development, from the region occupied by blastomeres B2, 3, 4 and
C2, 3, 4 at the 32-cell stage, which becomes the ventral and lateral marginal zone of the blastula (Dale & Slack, 1987a; Moody, 1987). But our explanation experiments have shown that this tissue is not specified to form muscle until after the beginning of gastrulation. Before this stage explants from the ventrolateral 270° of marginal zone circumference form ventral-type mesoderm with few, if any, muscle cells (Dale & Slack, 1987b). Ventral marginal explants will, however, form normal, or more than normal, amounts of muscle when cultured together with the organizer. We have previously called this phenomenon ‘dorsalization’ (Slack & Forman, 1980; Smith et al. 1985; Dale & Slack, 1987b), and the results presented above suggest two possible explanations for this process. First, it might be due to a second exposure of the prospective somite cells to a high local concentration of FGF in the dorsal region or, alternatively, the organizer may emit a distinct chemical signal with dorsalizing consequences, which can be mimicked by LiCl or TGFβ.

For the first possibility, our present results show that high doses of FGF alone can lead to the formation of substantial amounts of muscle, this is about 24% of the cells, and presumably a higher proportion of those exposed on the blastocoelic surface of the explant. We know from fate mapping that 20–50% of cells derived from blastomeres B2, 3, 4 and C2, 3, 4 become muscle and if this were solely due to FGF these cells would have been exposed to a concentration of 100–200ngml⁻¹ during dorsalization. The most-likely way this could be achieved would be for most of the endogenous FGF to be sequestered to extracellular material in the dorsal marginal zone. Then, as prospective somite cells migrate dorsally during gastrulation, they would encounter this high concentration and a sufficient proportion of them become specified as muscle. A dorsal concentration of toluidine blue metachromatic extracellular material has been detected in Rana blastulae by Johnson (1977), and it has been shown by Smith et al. (1982) that FGF can remain active when bound to cellular material has been detected in blastulae (Dickson & Peters, 1987). Moderate sequence homology to human TGFβ and that the corresponding endogenous molecule is the product of the vegl gene which shows a moderate sequence homology to human TGFβ (Weeks & Melton, 1987). It may be that both explanations are correct and that the organizer does indeed emit a TGFβ-like factor which provokes ECM secretion and hence local sequestration of FGF, although the published evidence suggests that there is no preferential dorsal location for the vegl message. A stimulation of ECM secretion by TGFβ is well documented in other systems (Massague, 1987).

The dorsalizing role of LiCl on VMZ explants is consistent with its effects on whole embryos (Kao et al. 1986; Cooke & Smith, 1988; our Fig. 4) which result in an overrepresentation of the anterior parts of the later embryo. Since the anterior parts normally arise from the dorsal region of the blastula, the implication is that the dorsal territories become larger than usual in whole embryos treated with LiCl. The actual mechanism of Li action at the molecular level is still not clear. Most current interest has centred on the inhibition of inositol monophosphatase (Hallcher & Sherman, 1980) which blocks the recycling of InsP₃ to inositol. In brain this depresses the effects of factors acting via InsP₃ by inositol depletion, but in other cell types it might perhaps enhance them by slowing the breakdown of InsP₃ or by potentiating the formation of other inositol polyphosphates with second message activity (Drummond, 1987). However, neither FGF nor TGFβ are known to act via this pathway (Magdalno et al. 1986) and a number of other biochemical effects of lithium have been described including an inhibition of G proteins (Avissar et al. 1988), competition with Na for Na channels, competition with Mg for Mg-requiring enzymes, inhibition of adenyl cyclase and increased turnover of catecholamines (Hendler, 1978), so it is not really possible at present to make deductions about the biochemical responses to induction based on the lithium effect alone.

In summary, the endogenous FGF of Xenopus embryos seems a good candidate as the sole agent for the induction of ventral mesoderm. It is probably also required for the induction of muscle from ventral mesoderm, perhaps together with a second factor. We have also shown that the FGFs and LiCl have inductive effects on a quite different type of amphibian embryo, the axolotl (J.M.W.S. unpublished data), suggesting that these results may be generalizable to other amphibian early embryos. At present there is little evidence allowing us to speculate on whether HBGFs are also morphogens in higher vertebrate embryos, although one of the three factors that we have shown to possess inducing activity is ECDGF derived from murine embryonal carcinoma cells (Heath & Isaacke, 1984), and the oncogene int-2, which is expressed in the mesoderm of the early mouse embryo, has a strong sequence homology to both forms of FGF (Dickson & Peters, 1987).

We should like to thank Dr A. Willis (MRC Immunology Lab., Oxford) for the amino acid composition analyses and Mr M. Wormald (Inorganic Chemistry, Oxford) for arranging the lithium measurement.
References


(Accepted 8 April 1988)