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Mesoderm Induction in Amphibians: The Role of TGF-β2–Like Factors

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Mesoderm induction in the amphibian embryo can be studied by exposing animal region explants (destined to become ectoderm) to appropriate stimuli and assaying the appearance of mesodermal products like α-actin messenger RNA. Transforming growth factor β2 (TGF-β2), but not TGF-β1, was active in α-actin induction, while addition of fibroblast growth factor had a small synergistic effect. Medium conditioned by *Xenopus* XTC cells (XTC-CM), known to have powerful mesoderm-inducing activity, was shown to contain TGF-β–like activity as measured by a radioreceptor binding assay, colony formation in NRK cells, and growth inhibition in CCL64 cells. The activity of XTC-CM in mesoderm induction and in growth inhibition of CCL64 cells was inhibited partially by antibodies to TGF-β2 but not by antibodies to TGF-β1. Thus, a TGF-β2–like molecule may be involved in mesoderm induction.

INDUCTIVE INTERACTIONS ARE CRITICAL to the elaboration of the body plan of all vertebrate embryos, as studied most extensively in amphibians. The earliest known induction concerns the establishment of the mesoderm (*1*). In normal embryogenesis the mesoderm develops mostly from cells that occupy a torus around the equator of the blastula, while cells from the animal hemisphere ("animal caps") primarily generate ectodermal derivatives (*1, 2*). Only ectodermal differentiation occurs after culture of animal caps derived from blastulae; mesodermal derivatives arise when animal caps are cultured in contact with vegetal tissue, implying an inductive interaction in mesoderm differentiation (*1*). Such interactions almost certainly also occur in normal embryogenesis (*3, 4*).

As shown by Smith (*5*) medium conditioned by the *Xenopus* XTC cell line (XTC-CM) is able to induce animal caps to form various mesodermal derivatives including muscle. Subsequently, Slack *et al.* (*6*) reported that fibroblast growth factor (FGF) has inducing activity, yielding various products including muscle (a dorsal mesodermal tissue), but primarily inducing ventral mesoderm, such as blood cells. Kimelman and Kirschner (*7*) reported that transforming growth factor β (TGF-β) potentiates the effect of FGF in inducing α-actin (indicating formation of muscle tissue).

TGF-β is known to occur in two related forms (*8, 9*). To further study the relationship of these factors to mesoderm induction we incubated animal explants from *Xenopus laevis* blastula embryos in medium containing TGF-β1 or TGF-β2 (*10*). TGF-β2 was found to induce mesoderm according to the following two criteria. In the presence of TGF-β2, animal explants formed elongated structures (Fig. 1A) reminiscent of mesodermal derivatives of the marginal zone and similar to those obtained with the mesoderm-inducing XTC-CM (Fig. 1B) (*5*). Further, TGF-β2 induced the expression of α-actin messenger RNA (mRNA) in the explants (Fig. 2A). During *Xenopus* embryogenesis, muscle-specific α-actin mRNA accumulates exclusively in the somites from stage 13 to 14 onward, providing the earliest known molecular marker for the differentiation of muscle (*11*). The lowest concentration of TGF-β2 that induced detectable α-actin mRNA varied from 3 to 12 ng/ml in different experiments; mRNA levels continued to increase until the TGF-β2 concentration was raised to about 200 ng/ml. The maximal level of α-actin mRNA induced by TGF-β2 was significantly lower than that found in heat- or acid-activated XTC-CM–treated animal explants (Fig. 2A). In agreement with other reports (*6, 7*), we found that TGF-β1 did not exhibit detectable mesoderm-inducing activity (Fig. 2A).

FGF exhibits weak muscle inducing activity (*6*), and it has been reported that this activity is potentiated by TGF-β (*7*). We tested α-actin induction by combinations of FGF with TGF-β1 or TGF-β2. TGF-β2 and FGF induced α-actin mRNA effectively, but the maximal level of mRNA obtained was the same as with TGF-β2 alone; the only effect of combining the two factors was a shift in the TGF-β2 dose response (Fig. 2B). In contrast, a moderate and somewhat variable enhancement was seen in the interaction between FGF and TGF-β1: the combination of these factors led to between two and three times higher α-actin induction than FGF alone, while TGF-β1 alone had no effect (Fig. 2, A and B).

Since XTC-CM (*5*) is a homologous material and has the highest inducing ability in these experiments, we wished to test whether the activity in XTC-CM and TGF-β2 (Figs. 1 and 2) might be related. Two approaches were taken. First, XTC-CM was assayed for TGF-β–like activity by several different methods; these assays do not differentiate between TGF-β1 and -β2 . Second, polyclonal blocking antibodies specific against TGF-β1 or -β2 were tested for their ability to inhibit both the TGF-β–like activity as well as the mesoderm-inducing activity of XTC-CM.

By means of a competitive radioreceptor binding assay (*12*), it could be shown that XTC-CM competed weakly for the binding of ^{125}I-labeled TGF-β1 (Fig. 3A). Pretreatment of XTC-CM with acid, a treatment known to activate latent TGF-β (*13*), resulted in a ninefold enhancement of the competing activity of XTC-CM; acid-treated XTC-CM was estimated to contain 220 p*M* (5 ng/ml) TGF-β activity, by comparison to the standard (Fig. 3A). Although purified TGF-β is intrinsically active, TGF-β secreted by many different human or rodent cell lines is in a latent form which is unable to bind to

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cellular receptors (*14*); this latent form can be activated by acidification of the medium (*13*). The parallel enhancement by transient acidification of both assayable TGF-β–like activity and mesoderm-inducing activity in XTC-CM strongly suggests that these two activities are related.

The ability to induce NRK cells to form colonies in soft agar in the presence of epidermal growth factor (EGF) is the classic assay of TGF-β activity (*15*). As shown in Fig. 3B, acid-activated XTC-CM stimulated the formation of large colonies of NRK cells; comparison with a TGF-β1 standard gave an estimated value of 160 p*M* TGF-β–like activity in XTC-CM.

TGF-β is a potent inhibitor of the growth of CCL64 cells (*16*). In our assay (*17*), acid-activated XTC-CM inhibited the growth of the cells similarly to human TGF-β1 used as a standard, with an estimate TGF-β concentration of 220 p*M* in XTC-CM (Fig. 3C).

Polyclonal antibodies raised in turkeys against either porcine TGF-β1 or TGF-β2 (anti–TGF-β1 or anti–TGF-β2) were used in an additional test of the structural relationship between the activity in XTC-CM and mammalian TGF-β. The specificity of the antibodies was tested with authentic TGF-β1 and -β2 in the CCL64 cell assay (Fig. 4): anti–TGF-β2 inhibited the activity of TGF-β2 completely and of TGF-β1 slightly, while anti–TGF-β1 was fully specific. Finally, to test whether the mesoderm-inducing activity of XTC-CM was dependent on the presence of a TGF-β–like peptide, the same blocking antibodies were used in an attempt to interfere with the induction of α-actin mRNA in animal cap explants. Antibodies against TGF-β2 inhibited the activity of XTC-CM by 80% (as measured by the level of α-actin mRNA accumulation), whereas antibodies against TGF-β1 had little effect (Fig. 4). In other experiments the range of blocking of α-actin accumulation by anti–TGF-β2 was between 60 and 80%. The fact that blocking is not complete may be due to imperfect recognition between the XTC-CM factor and the heterologous antibody. This result, together with the demonstrated presence of TGF-β–like activity in XTC-CM, indicates that at least one component of the mesoderm-inducing activity found in XTC-CM is structurally closely related to TGF-β2.

Our data lead to three conclusions. First, TGF-β2 is active in mesoderm induction, whereas TGF-β1 is not. Our observations confirm the conclusion of Kimelman and Kirschner (*7*) that TGF-β–like activity appears to be involved in the formation of mesoderm; however, unlike these authors

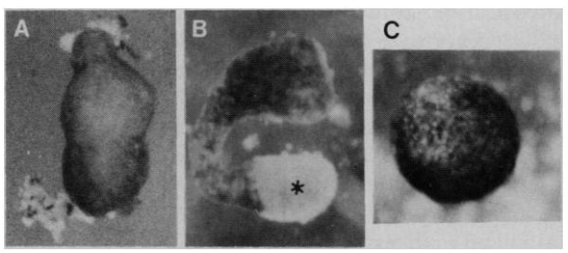

Fig. 1. TGF-β2 induces elongation of animal explants. Animal cap explants (*10*) incubated in TGF-β2 (50 ng/ml) (**A**) take on an elongated shape similar to that induced by incubation with XTC-CM (**B**). Note that the extrusion of the inner unpigmented layer (asterisk) obtained in XTC-CM is not observed with TGF-β2. Control (**C**).

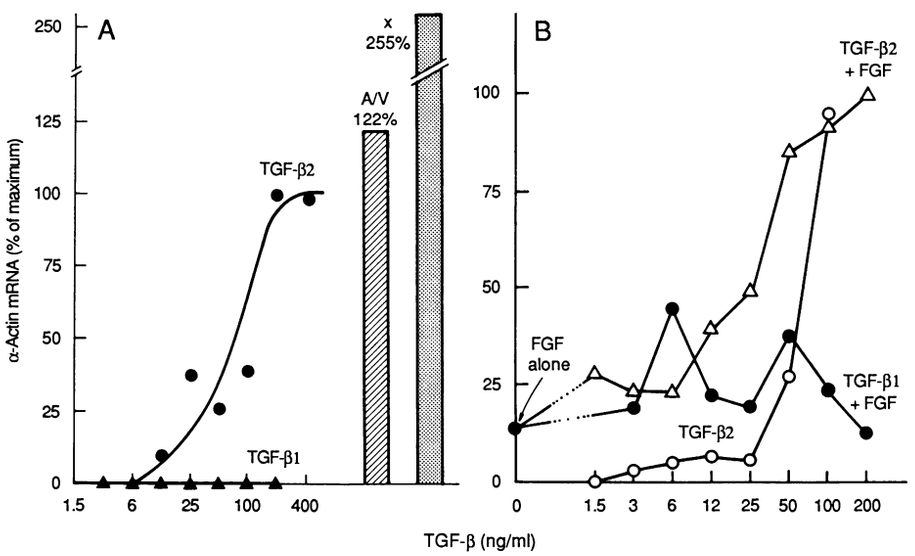

Fig. 2. TGF-β2 has mesoderm inducing activity as measured by accumulation of α-actin mRNA. (**A**) Tracing of a Northern blot showing induction of α-actin mRNA in animal caps (*10*) cultured with the additions shown. RNA was extracted and probed with an α-actin cDNA; muscle-specific α-actin mRNA visualized by radioautography and quantitated by densitometry (*3*). A/V is an animal cap/vegetal recombinate (*1*), x is an animal cap induced by XTC-CM. (**B**) Interaction between FGF and TGF-β in mesoderm induction. FGF concentration was 50 ng/ml which gives approximately maximal α-actin induction. The effect of TGF-β2 alone is shown from a different experiment than the one shown in (A).

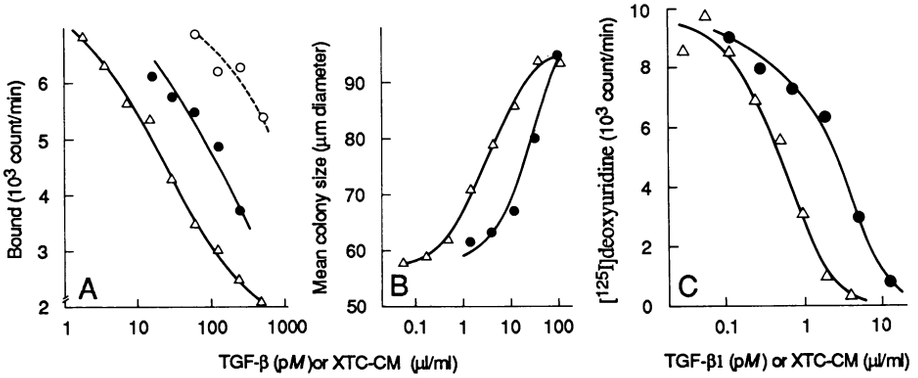

Fig. 3. XTC-CM contains a TGF-β–like activity. (**A**) Assay of the ability of XTC-CM to compete with ^{125}I-labeled TGF-β1 for binding to A549 human lung carcinoma cells (*12*), with (●) and without (○) prior acidification (*17*); TGF-β1 standard (△). (**B**) Assay of the ability of acid-activated XTC-CM to induce the formation of colonies of NRK cells in soft agar in the presence of 0.8 n*M* EGF (*15*) showed that XTC-CM (●) induced a dose-dependent increase in mean colony size analogous to human TGF-β1 (△). (**C**) Dose-response curves of the growth inhibition of CCL64 cells by human TGF-β1 (△) and acid-activated XTC-CM (●).

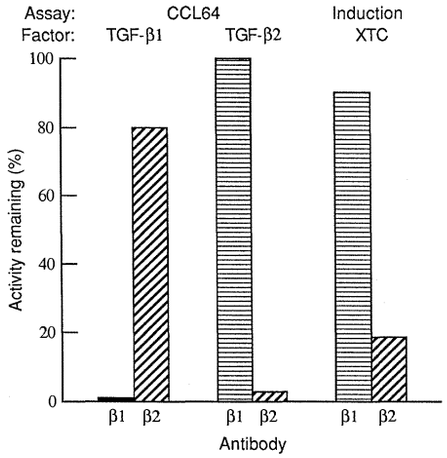

Fig. 4. Antibody blocking experiments. Each pair of bars shows the activity remaining after addition of anti–TGF-β1 or anti–TGF-β2 antibody to an assay system (*22*). The left two experiments are specificity tests, in which the CCL64 cell assay was used (*17*) (Fig. 3). The right pair of bars shows that antibodies to TGF-β2 block the ability of XTC-CM to induce α-actin mRNA in animal explants (*10*). Heat-activated XTC-CM was diluted 1/64-fold before treatment of animal caps. Dilution of the XTC-CM was necessary to see the antibody effect; at this concentration the level of α-actin RNA in the absence of antibody (100% in the figure) was 26% of the maximal level obtainable at optimal concentrations of XTC-CM.

we do not find significant synergism between FGF and TGF-β1, possibly because the two groups used different factor preparations. This issue and other differences in experimental design remain to be explored. The major result of the present work is that TGF-β2 alone is active in muscle induction, while the role of FGF appears more important in formation of ventral mesoderm (*6*). In most other biological assay systems TGF-β1 and -β2 act interchangeably (*8*), but it has been found recently that TGF-β1 can act selectively in inhibiting proliferation of hematopoietic progenitor cells (*18*), and we report here a selective action of TGF-β2.

The second conclusion is that XTC-CM, the most effective mesoderm inducing activity presently available, contains TGF-β–like activity as measured in several assay systems. We and others (*5*) have shown that these TGF-β–like activities, as well as mesoderm-inducing activity, are latent in XTC-CM, a typical property of TGF-β (*13*). Our third conclusion is that both the growth inhibitory activity of XTC-CM on CCL64 cells and the α-actin–inducing activity in embryo explants is inhibited by anti–TGF-β2, but very poorly by anti–TGF-β1 antibody (Fig. 4). This observation provides the strongest evidence for the TGF-β2–like nature of the active principle in XTC-CM.

Our results raise the following question: is the mesoderm-inducing activity in XTC-CM simply frog TGF-β2? Clearly, XTC-CM is much more effective in this system than mammalian TGF-β2. At least three possibilities arise. First, the active principle in XTC-CM is the *Xenopus* homolog of TGF-β2, but the mammalian factor has diverged sufficiently from the frog factor to diminish activity. Second, the XTC-CM factor is related to TGF-β2 but is not its direct homolog. Thus XTC-CM would contain a factor that represents a distinct member of the TGF-β family more closely related to TGF-β2. This explanation is compatible with all facts known at present. Third, the active principle in XTC-CM is a mixture or complex of *Xenopus* TGF-β2 and additional material which potentiates its activity.

Is the active principle in XTC-CM the natural mesoderm inducer? XTC cells were derived from a metamorphosing tadpole (*19*), a stage of development many weeks after mesoderm induction occurs. Inducing factors have been isolated previously from various sources; the factor of Tiedemann (*20*) has many properties reminiscent of TGF-β. A gene, *Vg1*, whose transcript is localized in the vegetal region of the *Xenopus* egg has been isolated and shown to be related in sequence to TGF-β (*21*). The product of *Vg1* is an excellent candidate for a factor involved in mesoderm induction in *Xenopus*. At present, the nature of the natural mesoderm inducer remains unresolved, but it appears highly probable that at least one critical component in this system is a molecule structurally related to TGF-β2.

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10. Animal caps were dissected from embryos at stage 8 to 8.5 (*5*) and cultured in L-15 Medium (Gibco) that had been diluted to 67% and contained BSA (1 mg/ml), penicillin and streptomycin at 10 μg/ml each, and 7.5 m*M* tris, *p*H 7.5. Growth factors or XTC-CM were added as indicated for the entire culture period. Explants were cultured for 20 hours until sibling embryos reached stage 24 to 25. RNA was extracted, separated by gel electrophoresis, and hybridized to an α-actin probe as described (*3*). XTC-CM was activated by heat (*5*); in other experiments, XTC-CM was activated by acid treatment (*13*) and gave similar results. TGF-β1 was purified from human platelets as described by Assoian *et al.* [R. K. Assoian, A. Komoriya, C. A. Meyers, D. M. Miller, M. B. Sporn, *J. Biol. Chem.* **258**, 7155 (1983)] and quantitated by amino acid analysis; porcine TGF-β2 (*8*) was purchased from R&D Systems, Minneapolis, MN. Basic FGF was obtained from Collaborative Research, Bedford, MA.
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17. TGF-β–like activity in XTC-CM was assayed as follows: XTC-CM was activated by acidification to 150 m*M* HCl followed by reneutralization as described (*14*). A competitive radioreceptor binding assay with A549 human lung carcinoma cells (*12, 14*) and an assay measuring the formation of colonies of NRK cells in soft agar in the presence of EGF (*15*) were carried out. Assay of the inhibition by TGF-β of the growth of CCL64 mink lung epithelial cells (*16*) was modified as follows: Cells were seeded into 24-well multidishes at a density of 5×10^4 cells per well in 0.5 ml 0.2% fetal bovine serum in Dulbecco's minimum essential medium. One hour later, TGF-β or XTC-CM (or antibodies, when appropriate) were added and the incubation continued for 22 hours. [^{125}I]deoxyuridine (Amersham; 0.5 μCi per well) was then added for an additional 2 hours incubation. After fixation in methanol:acetic acid (3:1), cells were washed, dissolved in 1*N* NaOH, and counted in a gamma counter.
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22. Antibodies were produced in turkeys by injecting porcine TGF-β1 or TGF-β2 (100 μg) in Freund's complete adjuvant and boosting every 2 weeks with equivalent amounts of TGF-β in incomplete Freund's adjuvant. Antisera were titered in an enzyme-linked immunoadsorbent assay and measured for blocking activity in either a radioreceptor binding or growth inhibition assay (*13*). In mesoderm induction assays (Fig. 4) antisera at 1:100 dilution were incubated with appropriately diluted XTC-CM for 1 hour at 23°C before addition of animal caps. Subsequent culture and processing was as in (*10*).

23. We thank L. Stuart and J. Poole for assistance in preparation of the turkey antibodies, and D. Melton and M. Kirschner for sharing unpublished information.

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