Disruption of mesoderm and axis formation in fish by ectopic expression of activin variants: the role of maternal activin

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Formation of mesoderm in *Xenopus* embryos is the result of an induction event in which peptides such as FGF or activins have been implicated. It was recently demonstrated, by the ectopic expression of a truncated activin receptor, that activin receptor signaling pathways are involved in the processes of mesoderm and axis formation in vivo. However, this approach does not directly address the role of activin itself nor the involvement of activins in the formation of mesoderm in embryos from other vertebrates. In addition, activins are expressed maternally as a protein component of the egg as well as transcribed zygotically, and it is not clear which of the two forms is involved in mesoderm formation. To address those three issues, we analyzed the role of activins in the development of fish embryos by generating two activin dominant-negative variants. One of the variants behaves as an inhibitor of activin protein. The second variant was found to deplete the activin pool when cotranslated with wild-type activin. Injection of RNA encoding these variants into the two-cell embryo of the small teleost fish *Oryzias latipes* (Japanese medaka) demonstrates that only the maternally provided activin protein is required for mesoderm and axis formation in fish in vivo.

[**Key Words:** Medaka; zebrafish; mesoderm induction; activin; dominant-negative variant]

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Vertebrate embryos rely heavily on cell–cell interactions to generate a structured animal from a radially symmetric egg. In particular, induction is the process during which a population of cells emits a signal that will change the fate and/or behavior of neighboring target cells. In amphibian embryos, mesoderm derives from the marginal zone ectoderm under the influence of inducing signal(s) emanating from the endoderm (Nieuwkoop 1973). Mesoderm, in turn, plays an important role in the organization of the body axis. The dorsal region of the newly induced germ layer, the organizer, can induce a secondary body axis after transplantation in amphibia (Spemann and Mangold 1924) and in fish (Luther 1935). Analysis of mesoderm induction for the last 70 years suggested that at least three types of signals emerging from the vegetal half of the embryo or localized in the marginal zone are required for mesoderm induction (for review, see Kimelman et al. 1992). The nature of a signal localized in the marginal zone is probably a factor with a Wnt-like activity (for review, see Kimelman et al. 1992) or is *noggin* (Smith and Harland 1992). These factors do not induce mesoderm (Smith and Harland 1991, 1992) but may act as dorsalising potentiators (Smith et al. 1993). All of them rescue normal axis formation in *Xe-*

nopus embryos ventralized by UV irradiation during the first cell cycle.

Polypeptide growth factors encoded by two different gene families, the fibroblast growth factor (FGF) family (Kimelman et al. 1988; Slack et al. 1987; Isaacs et al. 1992) and the transforming growth factor- β (TGF- β) family [activin (Asashima et al. 1990; Smith et al. 1990; Vale et al. 1990); bone morphogenetic protein 4 (BMP-4; Köster et al. 1991); Vg1 (Weeks and Melton 1987)] formally fulfill most of the criteria for a mesoderm inducer. The genes encode proteins carrying a signal peptide for secretion. Either mRNA [XeFGF (Isaacs et al. 1992); BMP-4 (Köster et al. 1991; Dale et al. 1992; Jones et al. 1992)] or proteins [FGF (Slack et al. 1987; Kimelman et al. 1988); activins (Asashima et al. 1991; Ge et al. 1993a); Vg1 (Weeks and Melton 1987)] have been detected prior or coincident with mesoderm induction in vivo (as maternal and/or zygotic components). In addition, Vg1 mRNA is localized in the prospective endoderm (Weeks and Melton 1987). XeFGF, activin, and BMP-4 induce mesodermal structures in vitro or induce the formation of axial structures upon injection of their RNA into ventral blastomeres of the early *Xenopus* blastula. The carboxy-terminal part of Vg1 will induce mesoderm only if properly processed, as suggested by the use of BMP–Vg1 chimeric RNA (Dale et al. 1993; Thomsen and Melton 1993).

Quite strikingly, activins induce a wide variety of me-

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soderm derivative in a dose-dependent fashion. Sharp thresholds of activin concentrations induce the distinct expression of mesodermal markers in *Xenopus* animal cap cells. Low concentrations of activin induce the expression of ventral–posterior markers whereas high concentrations induce dorsal–anterior markers (Green et al. 1992). The presence of an activin concentration gradient along the dorsoventral axis of the amphibian embryo endoderm would be sufficient to explain the inducing properties of the early endoderm and its involvement in the patterning of the primary mesoderm as suggested by Green et al. (1992).

The involvement of activins and FGF in mesoderm induction in vivo has been analyzed by interfering with downstream components of the signaling pathways, for example, with their receptors. Injection of RNA encoding a dominant-negative FGF receptor into *Xenopus* embryos prevented the formation of ventral–posterior mesodermal structures, suggesting that a member of the FGF family serves as an inducer for ventral posterior mesoderm (Amaya et al. 1991). As well, injection of high amounts of a truncated form (Hemmati-Brivanlou and Melton 1992) of one of the four known *Xenopus* activin receptors (XAR1; Nishimatsu et al. 1992) led to a significant reduction in the expression of mesodermal structures and markers, including the most dorsal–anterior ones (Hemmati-Brivanlou and Melton 1992). This suggests a role for the XAR1 activin receptor signaling pathway in the establishment of mesoderm. Although other activin receptors are expressed maternally, their involvement remains unclear (Nishimatsu et al. 1992).

Experiments relying on truncated receptors provide only indirect evidence for FGF or activin involvement, as these receptors might interfere with related pathways as well. A direct analysis of the role of activins in vivo necessitates interference with the activity of the endogenous activins. This could be achieved either by deleting or mutating the endogenous genes or by a specific dominant-negative interaction (Herskowitz 1987) blocking the wild-type activity in the developing embryo. The latter approach could also distinguish between maternal versus zygotic activin contributions.

We chose a small freshwater fish, the Japanese medaka (*Oryzias latipes*) to investigate the role of activin in mesoderm induction and axis formation in lower vertebrates. In addition to the advantages known for other small egg-laying fish (e.g., fundulus, zebrafish), stable transgenics can be generated with medaka that express their transgenes after injection into the germinal vesicle (Ozato et al. 1986). Although mesoderm induction has not been analyzed in such detail in fish, the factors and the mechanism involved in embryonic induction in fish appear to be quite similar to those in higher vertebrates. The transplantation of the archenteron roof in trout induces the formation of a secondary body axis (Luther 1934). The zebrafish embryonic shield transplanted to *Triturus* blastulae acts as an organizer across species borders (Oppenheimer 1936). Expression of mesodermal markers, for example, of *Brachyury*, is localized in homologous regions of the fate map in fish and *Xenopus*. In

explanted animal caps the expression of *Brachyury*, as an immediate marker for mesoderm induction, is induced by activins in zebrafish (Schulte-Merker et al. 1993) as well as in *Xenopus* (Smith et al. 1991).

Activins are active as homo- or heterodimers formed after dimerization of the two known activin β -chains (activin β A and β B). To obtain their inducing potential, the highly conserved carboxy-terminal region must be cleaved off and then released from the amino-terminal remainder (for review, see Vale et al. 1990). On that basis, we generated dominant-negative fish activin variants. One variant inhibiting wild-type activin allowed us to determine the role of maternally provided activin protein. The role of the zygotically expressed activin was analyzed with a variant that acts upon cotranslation. The results of our experiments demonstrate that only the maternally provided activin protein is required for the induction of mesoderm and axis formation. Furthermore, embryos expressing an increasing dose of dominant-negative variant progressively lose dorsal–anterior structures.

Results

Isolation of fish activin cDNA

Partial sequences representing the carboxy-terminal portions of activin chains were amplified from medaka and zebrafish genomic DNA by the polymerase chain reaction (PCR). Clones highly homologous to activin β A (β A₁ and β A₂) and β B from higher vertebrates were obtained from medaka and zebrafish. The sequences of the activin peptides encoded by the PCR fragments homologous to β B are shown in Figure 1. The deduced medaka and zebrafish β B amino acid sequences share 92% identity. A zebrafish activin β B cDNA (Zact β B) was isolated from a cDNA library established from 1-day-old embryos. Zact β B encodes a protein with 73% overall identity to mammalian activin β B, including all of the evolutionary conserved residues. The encoded protein contains a putative signal peptide for secretion and a tribasic region preceding the conserved carboxyl terminus, which is expected to serve as a proteolytic processing site for the release of the active carboxy-terminal peptide.

Expression

The expression of the medaka activin β B gene was analyzed by Northern blot assays (data not shown). A 2.3-kb transcript was detected in ovary and testis as expected (Vale et al. 1990). No activin transcript could be found in early embryos. We therefore used reverse transcriptase–polymerase chain reaction (RT-PCR) to increase the sensitivity of detection. Expression of activin β B was assayed with specific primers that amplify a 264-bp fragment. Southern blots of the PCR products were hybridized with the corresponding medaka probe under high stringency. RT-PCR performed with RNA from staged embryos shows that β B mRNA first appears in late blas-

The second clue for the creation of variants derives from the dimeric nature of the active form. Targets for

Translation in *Xenopus* oocytes was used to assay the processing and interactions of the wild-type and variant activin. Wild-type activin is secreted and processed by the oocyte to generate a 15-kD peptide (carboxyl terminus) and a 41-kD peptide (precursor remainder) under reducing conditions (Fig. 4B,C; lanes wt red) as described for activin secreted from vaccinia virus-infected tissue culture cells [Huylebroeck et al. 1990]. Under nonreducing conditions, the 15-kD species is shifted to 25-kD in agreement with the dimeric structure of activin (Fig. 4B,C). A minor additional ~58-kD peptide was also detected under nonreducing conditions. When isolated from the gel and run under reducing conditions, it was

split into two peptides of 15 and 41 kD, respectively, comigrating with processed peptides of activin β B (data not shown). Most likely, the 58-kD peptide is the result of the formation of an unusual disulfide bridge between the precursor remainder and the carboxy-terminal part of activin β B, as a result of overexpression in the oocyte. Identical results were obtained with the Pt variant (Fig. 4D). On the contrary, CS protein is secreted as a single species of 58 kD (reducing conditions) and 120 kD (nonreducing), clearly demonstrating that the CS protein can no longer be processed but can still dimerize (Fig. 4B–D). Coinjection of RNAs encoding CS and wild-type or Pt activin β B leads, under nonreducing conditions, to the simultaneous depletion of the 25-kD band and the accumulation of a new species of $\sim$ 65 kD (Fig. 4B–D; non red.), a size expected from a heterodimeric CS/wt or CS/Pt molecule. When isolated from the gel and run under reducing conditions, the 65-kD species was split into a 15- and a 58-kD species, confirming its heterodimeric structure (data not shown). Therefore, although unprocessed, the CS variant can still dimerize with wild-type or Pt forms.

Analysis of the dominant-negative activity in vitro

Both CS and Pt variants inhibited activin β B mesoderm inducing activity when cotransfected with pC β B. Conditioned medium from COS cells cotransfected with pC β B and pCMV5 DNAs (ratio 2:1) strongly induces morphogenetic movements (data not shown) and cardiac actin expression (Fig. 5A,B). Replacing pCMV5 with pC β B–CS results (at the same ratio) in the strong inhibition of actin expression (Fig. 5A,B) and only minor elongation of the caps. Further increase of the ratio of variant DNA ($>50\%$) in the cotransfection leads to a supernatant that shows no mesoderm-inducing activity, whereas the corresponding controls significantly induce mesoderm (Fig. 5A,B). Pt variant similarly inhibited wild-type activity, although less efficiently (Fig. 5A,B).

To determine at which level Pt mutant was interfering with activin activity, we mixed media from cells transfected with either wild-type or variant activin plasmids and tested their activity on the induction of caps elongation (not shown) or Mix.1 (Rosa 1989) RNA (Fig. 5C,D). CS supernatant did not inhibit wild-type supernatant activity (Fig. 5C), supporting the idea that CS and wild-type activin must be cotranslated to interact. On the contrary, Pt inhibited the elongation of caps (not shown) and Mix.1 induction by activin β B (Fig. 5C,D), suggesting that Pt interferes directly with processed activin either by blocking receptor binding or by blocking the signal transduction pathway. Similar experiments suggested that the Pt variant is also able to inhibit activin β A (data not shown).

The specificity of the interaction of the CS and Pt variants with activin β B was tested by analyzing their capacity to interfere with the activity of BMP-4, another potential mesoderm inducer belonging to the TGF- β superfamily (Köster et al. 1991) in two different assays. In the first assay, BMP-4 is inducing the differentiation of

SAOS-2 osteogenic sarcoma cell line (Fogh and Trempe 1975) cells in vitro, as analyzed by the expression of alkaline phosphatase (Gray et al. 1987). Coexpression in COS cells of the CS or the Pt activin variant with *Xenopus* BMP-4 did not inhibit noticeably the capacity of *Xenopus* BMP-4 to induce differentiation of SAOS cells (Fig. 6A, mp). Similarly, adding supernatants from pC β B–Pt or pC β B–CS to supernatants from *Xenopus* BMP-4-transfected cells did not inhibit the differentiation of SAOS cells induced by BMP-4 (Fig. 6A, ms). In the second assay, BMP-4 RNA injection during the first cell cycle has been shown to ventralize *Xenopus* embryo mesoderm (Dale et al. 1992; Jones et al. 1992). In particular, animal caps from BMP-4-injected embryos express an increased level of the homeo box gene *Xhox3*, a marker for posterior mesoderm. If Pt variant is able to interfere with BMP-4 activity, it should inhibit the increase in *Xhox3* mRNA induced by ectopic expression of BMP-4. To test this, animal caps from control or BMP-4-injected *Xenopus* blastula were dissected and incubated in dilutions of conditioned media derived from 293 cells expressing either wild-type activin or Pt variant, and corresponding *Xhox3* mRNA levels were measured by RNase protection. BMP-4 injection led to increased levels of *Xhox3* mRNA (Fig. 6B). However, incubation of animal caps from BMP-4-injected embryos in Pt-conditioned medium did not markedly inhibit this increase (Fig. 6B; Table 1). In one experiment (involving high concentrations of Pt), levels of *Xhox3* were actually increased by incubation of the caps in Pt-conditioned medium. Such an increase could be interpreted to indicate that activin antagonizes the *Xhox3*-inducing capacity of BMP-4 and that the Pt variant, by blocking the effect of activin or activin receptor, amplifies or unmasks additional inducing activities of BMP-4. A similar observation was reported in the case of enhanced induction of *Xbra* by basic FGF (bFGF) in *Xenopus* embryos injected with truncated XAR1 receptor (Hemmati-Brivanlou and Melton 1992). In summary, both Pt and CS variants specifically inhibit activin activity by two different mechanisms. CS variant is able to titrate out wild-type activin, whereas Pt variant inhibits the processed protein.

Analysis of the role of activin in vivo

The involvement of activin in the processes of mesoderm induction and axis formation was addressed directly by injecting Pt RNA into one of the two blastomeres of the medaka embryo. For each type of injection, several series of experiments were performed. In total, >1500 embryos have been injected with different amounts of the variant RNAs and corresponding controls. The data shown in the figures are representative and have been taken from one series of experiments. Injected embryos were analyzed after 32 hr when controls had completed gastrulation and were at the 12-somite stage.

Embryos injected with high amounts (500 fg) of synthetic Pt RNA (high Pt embryos) exhibited a uniform phenotype. After 32 hr all cells of the embryo were lo-

Figure 5. In vitro analysis of dominant-negative activity of the variant activin proteins. (A) CS and Pt variants inhibit actin induction in *Xenopus* animal caps when cotransfected with wild-type activin. Stage 8 *Xenopus* animal caps were incubated with supernatants from cells transfected at the indicated ratio with pCMV5 (control), pCβB (wt), pCβB-Pt (Pt), pCβB-CS (CS), or a combination of those and incubated until control embryos reached stage 17. RNA was extracted from caps and analyzed by Northern blotting with an α -actin probe (top). XTC medium was used as a control for induction (right lane). The autoradiography is shown. As a loading control, the filter was stained with methylene blue, and 18S rRNA staining is displayed (bottom). (B) Quantitation of the effect of the Pt and CS variants shown in A. Induction is shown as expression relative to the maximum effect observed when caps were incubated with supernatant from pCβB-transfected COS cells. The curve represents an average of three different experiments. Error bars are indicated. (C) Pt activin inhibits the effect of wild-type activin on Mix.1 induction in *Xenopus* animal caps. RNA was prepared from animal caps incubated in conditioned media from 293 cells transfected with pCβB (wt), pCβB-Pt (Pt), pCβB-CS, or a mixture of the supernatants and incubated until control reached stage 10.5 (early gastrula). In this experiment, Pt supernatant concentration was kept constant and mixed with varying dilution of supernatant from pCβB-transfected cells (dilution indicated above). (Con) Control medium. RNA from two caps was hybridized with a Mix.1 RNA probe and a probe for Ef-1 α RNA and processed for RNase protection as described in Materials and methods. (Top and middle) A long (6-day) and a short (16-hr) exposure, respectively, of the same gel (Mix.1). (Bottom) The expression of Ef-1 α . The relative weakness of the effect observed might come from the lower level of Pt protein present in the medium (data not shown). (D) Quantitation of the effect of Pt on wild-type activin shown in C. Induction was normalized to the amount of Ef-1 α RNA and plotted as expression relative to the maximum effect observed when caps were incubated with supernatant from pCβB-transfected 293 cells (wt+con). The curve represents an average of two independent experiments. Induction of Mix.1 obtained at dilution 1/32 and 1/64 is too low to be reproducibly quantitated. Error bars are indicated. The effect of CS variant was studied in independent experiments and not represented on the curve. Quantitation showed that mixture of wild-type and CS activin induced Mix.1 RNA to 98% of the level induced by wild-type activin alone.

cated at one pole of the yolk sack forming an unstructured "cap." No obvious axis had been formed (Fig. 7A). Time lapse video microscopy revealed that at the time when controls started to gastrulate, cells from the high Pt blastulae did not show any obvious gastrulation movements. Cells were still alive, as methylene blue contained in the medium was efficiently excluded from the embryos. The rate of dead embryos was not signifi-

cantly increased (Fig. 10A, below). Histological analysis revealed only two types of cells, a single layer of cells with flat nuclei covering the yolk, presumably the yolk syncytial layer and a multilayer region (the cap) composed of a rather uniform population of cells reminiscent of blastula undifferentiated blastomeres (Fig. 7B). Reduction of the amount of Pt variant RNA injected (100 fg; intermediate Pt) led to a shift in the predominant phe-

Figure 6. (A) CS and Pt activin do not interfere with BMP-4-induced differentiation of SAOS cells. SAOS-2 cells were incubated for 36 hr in medium conditioned by COS cells cotransfected with constant amount of variant or control DNA (CS, Pt, pCMV5) and indicated dilution of BMP-4 [mixed plasmids (mp)]. Alternatively conditioned media from COS cells transfected with BMP-4 and the activin variants or control (CS, Pt, pCMV5) were mixed and applied to SAOS cells under identical conditions. A constant amount of variant or control and indicated dilution of BMP-4-conditioned medium was used [mixed supernatants (ms)]. Control supernatants (right) were derived from COS cells transfected with respectively pCβB-CS, pCβB-Pt, pCMV5, or left untreated, respectively. Alkaline phosphatase activity is plotted vs. the plasmid used. (B) Pt treatment does not inhibit *Xhox3* induction in animal caps from BMP-4-injected *Xenopus* embryos. Stage 8 animal caps were dissected from control or BMP-4 RNA-injected embryos and incubated in medium indicated: (con.) Control; (wt) wild-type activin; (wt 1:10) same at dilution 1:10; (Pt) Pt variant; (Pt 1:10) same at dilution 1:10. Caps were harvested when sibling embryos reached stage 12 and analyzed for their content of *Xhox3* mRNA by RNase protection.

notype. Most of the embryos (55%; Fig. 10A, below) were forming some structures like somites (Fig. 7C, cap with structure, arrowhead). Although no sign of proper axis formation was found in those embryos, mesodermal derivatives such as red blood cells were consistently observed after long-term incubation of the int Pt embryos. Pt embryos injected with 25 fg of Pt RNA (low Pt) expressed distinct morphological structures. The most prominent phenotype in this group (Fig. 7D) (reduced axis; 48% of all injected embryos; Fig. 10A) contained an axis with obvious deficiencies in the anterior part such as lack of head or eyes and fore-/midbrain. Interestingly,

a minor fraction of the low Pt embryos (3%, Fig. 10A) exhibited the formation of twin tails. In summary, Pt RNA injection led to a progressive, dose-dependent loss of axial structures. Strikingly, embryos injected with a given RNA amount exhibited a rather narrow range of phenotypes.

Antibodies specifically recognizing mesodermal structures were used to refine the phenotypical and histological analysis. Notochord, a dorsal mesoderm derivative, was identified with mAb MZ 15 (Zanetti et al. 1985). Staining in the wild-type embryo was first detected at 24 hr in the posterior notochord, extending from the tailbud to anterior as development proceeds (Fig. 8A,D). Brachyury protein (T protein), expressed in immediate response to mesoderm induction in frogs (Smith et al. 1991) and fish (Schulte-Merker et al. 1993), was used as a general marker for mesoderm. Its expression pattern in wild-type medaka embryos (Fig. 8B,E) studied with an antiserum, kindly provided by S. Schulte-Merker, is very similar to the expression described for zebrafish (Schulte-Merker et al. 1993).

High Pt embryos did not express detectably MZ 15

Table 1. Effect of activin/Pt variant on *Xhox3* induction by BMP-4

Injection	Treatment	Experiment 1 induction by BMP-4	Experiment 2 induction by BMP-4
Control	control	1.00	1.00
BMP-4 sense	control	3.83	2.73
BMP-4 sense	wild-type activin	3.70	1.51
BMP-4 sense	wild-type activin 1/10	2.94	2.17
BMP-4 sense	Pt variant	2.64	6.04
BMP-4 sense	Pt variant 1/10	3.15	3.76

The gels from Fig. 6 (experiment 1, wild-type ≈ 10 U/ml) and from another experiment carried out with 10-fold concentrated supernatants (experiment 2) were quantitated using a Molecular Dynamics PhosphorImager device. Treatment indicated in column 2 is described in Fig. 6. RNA amounts were normalized with regard to *Ef-1α* levels. Relative induction levels (*R*) shown in the two right columns were calculated according to the formula

$$R = \frac{\text{Amount of } Xhox3 \text{ mRNA in treated caps from injected embryos}}{\text{Amount of } Xhox3 \text{ mRNA in treated caps from uninjected embryos}}$$

In experiment 1, Pt supernatant inhibits slightly *Xhox3* induction, but this effect was not observed with a more concentrated supernatant (experiment 2; Fig. 6, cf. lanes 5 and 6 with lane 2). In experiment 2, wild-type activin clearly inhibits *Xho3* induction. This supernatant has a 10-fold higher inducing activity than the one used by Dale et al. (1992) and might override BMP-4 effect. In this sample, animal caps exhibited strong pseudogastrulation movements typical for activin-mediated mesoderm induction.

Figure 7. Pt RNA injection disrupts axis formation in medaka. Medaka embryos were injected at the two-cell stage into one of the blastomeres with different amounts of Pt RNA and kept at 28°C for 32 hr. (A) Caps are obtained after injection of 500 fg of Pt RNA. (B) The caps shown in A exhibit a blastula-like histology. (C) Injection of intermediate amounts of Pt RNA (100 fg) predominantly leads to the cap with structure phenotype, show here with a somite-like structure (arrowhead). (D) Low amounts of Pt RNA (25 fg) induce loss of anterior structures. (E) Control uninjected embryo. (F) Cross section through the trunk region of a control embryo. Diameter of the embryos is ~1 mm.

antigen or T protein (Fig. 8G,H). In caps with structure MZ 15 and T-protein antibodies labeled cells appearing in a connected cord. In contrast to normal embryos this staining was detected almost exclusively on the surface of the embryo rather than inside (Fig. 8J,L), suggesting that some mesoderm had differentiated without being able to gastrulate inside the embryo.

Some of the 32-hr intermediate Pt embryos looked like retarded gastrula (50% epiboly; Fig. 8K). However, none of them expressed the T protein as a complete ring as usually seen in wild-type 50% epiboly gastrula (Fig. 8B). Low Pt embryos with reduced axis in most cases showed T-protein expression anterior to the truncation of the axis (Fig. 8O), suggesting that the anterior part of the

embryo had acquired a posterior character as a result of Pt expression.

We conclude from these experiments that Pt RNA injection efficiently inhibits mesoderm induction in the medaka embryo and that activin is necessary for both mesoderm induction and axis formation in fish medaka.

Recent experiments suggested that in *Xenopus*, activin signaling inhibits the spontaneous conversion of ectoderm to neuroectoderm (Hemmati-Brivanlou and Melton 1992). In particular, animal caps from *Xenopus* embryos expressing high amounts of XAR1-truncated activin receptor express large amounts of neural cell adhesion molecule (N-CAM), a marker for early neural tissue. We investigated whether this result could be generalized to lower vertebrates. A monoclonal antibody (mAb D3; Schlosshauer 1989) cross-reacting with goldfish N-CAM (Bastmeyer et al. 1990), was used to identify neural tissue in injected embryos. In wild-type embryos, D3 detected N-CAM expression on the surface of neural tissue cells. It first appears at the neurula stage in the neural keel. In older embryos the entire central nervous system is stained (Fig. 8C,F). Animal caps were dissected from control of high Pt medaka embryos and stained with mAb D3. Because medaka gastrula fate map is ill defined, we relied on zebrafish gastrula fate map (Kimmel et al. 1990). Animal caps from control embryos did not exhibit obvious N-CAM expression (Fig. 9, cf. A and B). On the contrary, animal caps from high Pt embryos exhibited strong staining with mAb D3, suggesting that Pt ectopic expression led to elevated expression of N-CAM and neural tissue formation (Fig. 9F). Surprisingly however, only 55% of high Pt embryos did express N-CAM. However, this expression was restricted to small patches of cells (Fig. 8I). In intermediate Pt embryos, expression of N-CAM in cells organized in a tissue-like manner was clearly observed (Fig. 8M). Low Pt embryos with reduced axis showed abundant expression of N-CAM (Fig. 8P). In conclusion, although we observed enhanced expression of N-CAM in animal caps from medaka embryos injected with Pt RNA, injection of high amounts of Pt RNA did not result in the formation of large amounts of neural tissue in whole embryos, as would have been expected.

Maternal vs. zygotic contribution

In the early embryo, activin is found as a maternal protein (Asashima et al. 1991; Ge et al. 1993a; J. Wittbrodt and F. Rosa, this paper), whereas activin β B is transcribed zygotically (late blastula stage; J. Wittbrodt and F. Rosa, this paper). Both forms could be involved in mesoderm induction and axis formation. To analyze the role of zygotically expressed activin in vivo we used the property of the CS variant to titrate out activin upon cotranslation. CS variant mRNA was injected into each blastomere of the two-cell embryo. Stability of CS mRNA was tested by Northern blot analysis (Table 2). At a time at which the zygotic transcript of activin is first detected in the embryo (10–12 hr after injection), there is still 80

Figure 8. Pt RNA injection alters mesoderm and the expression of neural markers in medaka embryos. Embryos injected with varying amount of Pt RNA (*G–P*; amount injected indicated at *right*) or left uninjected (*A–F*) were collected after 32 hr (injected embryos and control embryos in *D,E,F*) at 28°C or after 24 hr (*A*), 16 hr (*B*), 18 hr (*C*), fixed and processed for whole-mount immunostaining with antibodies (indicated at *top*). (*G,H,I*) Cap embryos do not express MZ15 antigen or T protein but express (55%) a low level of N-CAM in a small patch of cells (arrowhead). (*J,K,L,M*) Cap with structure embryos express the three markers in a disorganized fashion. Note that in *J* and *L*, marker is expressed at the surface of the embryo (arrow in *L*). Occasionally, we observed a structure reminiscent of an early neural keel (arrowhead in *L*). (*N,O,P*) Reduced axis embryos. Note the anterior staining in *O* for T protein. N-CAM seems expressed more strongly in *P* than in wild-type embryos (cf. *F*). Dorsal view, anterior to the *left* (*A,C,D,L,M,P*); lateral view, anterior to the *left* (*E,f,N*); anterior view (*O*).

pg of CS RNA present in the blastula. This corresponds to at least a 100- to 1000-fold molar excess over the activin mRNA transcribed in the embryo, as we cannot detect the endogenous mRNA by Northern hybridization in embryos. The phenotype of the CS embryos is indistinguishable from control injected or uninjected sibling embryos throughout their entire development (Fig. 10B).

To demonstrate that the CS variant has the potential to titrate out activin *in vivo*, we coinjected it with wild-type activin mRNA. Zebrafish activin β B, as well as mouse β A RNA injection, leads to multiple axis formation at a high frequency (54% and 54%, respectively; Fig. 10B). In both cases, this effect is blocked by the coinjection of a 20- to 40-fold excess of the CS variant RNA (Fig. 10B).

Therefore, the activin involved in mesoderm induction and axis formation in medaka does not need to be translated prior to its use. A logical conclusion is that the activin involved is stored in the egg as a maternal protein.

Rescue of the effect of Pt

If the defects in axis formation and mesoderm induction observed in the Pt embryos are caused by the Pt variant protein, coinjection of an excess of wild-type RNA should rescue normal embryonic development. Coinjection of 2.5 pg of wild-type activin RNA and 500 fg of Pt RNA led to embryos exhibiting stronger epiboly movements (Fig. 10C,D). Coinjected embryos ($n=61$) reproducibly resumed T expression at the rim of the blastoderm as well, although immunostaining was weaker than T expression in the germ ring of wild-type embryos. However, small clusters of cells, isolated from the blastoderm and localized to the vegetal pole, exhibited strong staining. Coinjection did not lead to recognizable phenotypes in late embryos (data not shown), although some embryos with multiple axes were obtained ($n=4$ of 61—no embryo with multiple axes was found among high Pt embryos). There is one major caveat in this approach: Injecting activin RNA at the two-cell stage makes it likely that all cells of the embryo will be in

Figure 9. N-CAM expression is induced in animal caps isolated from Pt-injected medaka embryos. The animal cap region (or the marginal zone) from early blastula (stage 10) embryos was dissected and incubated in isolation until sibling embryos reached the 12-somite stage. Explants were then processed for immunohistochemistry with (+ D3) or without (– D3) mAb D3. Two independent experiments involved a total of 10 explants (A), 30 (B), 30 (C), 25 (D), 10 (E), and 30 (F) explants.

contact with wild-type activin. Therefore, unlike the experiments involving the use of a dominant-negative form of the activin receptor (Hemmati-Brivanlou and Melton 1992), we expect that all of the competent cells will be in contact with wild-type activin, resulting in nonorganized mesoderm (Cooke et al. 1987). On the other hand, a specific block of Pt should specifically rescue its phenotype. Although CS injection does not affect development, it can titrate out activin activity upon coexpression (Fig. 5A,B). By the same mechanism as demonstrated above (Fig. 4D), it should be able to block Pt activity in embryos. We used this property to test the specificity of the Pt effect on developing fish embryos. Pt and increasing amounts of CS mRNA were coinjected. The CS RNA rescues the effect of the Pt RNA as assayed

Table 2. Stability of CS–RNA after injection

Hours after injection	0	2	6	8	10	22	26	32
Intact CS–RNA (pg)	120	93	91	86	84	57	54	25

RNA was injected into both blastomeres of the two-cell embryo and embryos were allowed to develop. After the given time, RNA from five embryos for each time point was isolated. On Northern blots the amount of intact CS–RNA was quantitated in comparison to loaded controls. Onset of endogenous medaka activin β B expression is after 10 hr.

by the phenotype analysis. Analogous to the experiments described above, the phenotype observed depends on the dose of remaining Pt activity. At a 10-fold molar excess of CS over Pt (250 fg/25 fg) the number of normal embryos was increased from 12% to 33%. At the same time, the number of embryos with reduced axes were significantly decreased (from 48% to 21%) (Fig. 10E). An almost complete rescue (78% normal embryos) was obtained at a 20-fold molar excess of the CS RNA over Pt RNA.

Discussion

Partial sequences of the genes encoding activin β B and β A have been cloned by PCR from medaka and zebrafish genomic DNA. This confirms the presence of activin genes in fish, as initially reported in goldfish by Ge et al. (1993b). Activin β B was cloned as a full-length cDNA. β B mRNA is expressed both in the adult ovary and during embryogenesis at approximately the time of midblastula transition. With a monoclonal antibody to human activin, we show here that activin reactivity is present as a maternal component in the egg and persists until at least the blastula stage.

To elucidate the role of maternal and zygotic activin in mesoderm induction and axis formation *in vivo*, we have designed variant forms of the protein. Our experiments have demonstrated that the two types of variants specifically inhibit wild-type activin activity on explanted *Xenopus* animal cap cells by two different mechanisms. The CS variant inhibits activity only when coexpressed in the same cells with wild-type activin. Coexpression results in the formation of secreted heterodimers that cannot be processed and leads to the depletion of the wild-type activin pool. This type of dominant-negative mutation that is specific for activins and does not interfere with BMP-4 might be of general use throughout the TGF- β gene family. In transgenic animals a targeted expression and dominant-negative interaction with given TGF- β family members may permit the correlation of temporal expression patterns and the function of the factors analyzed.

The same is true for the Pt variant. In contrast to CS, the Pt variant behaves as an inhibitor of activin (β A and β B) protein. This may be attributable to Pt protein binding to the activin receptor without activating the signal transduction cascade. It could also be attributable to interaction with related receptors, with the wild-type activin itself, or with downstream components of the activin signaling pathway. The Pt variant shows high specificity in the interactions with activins and does not interfere with signaling pathways of distantly related members of the TGF- β family like BMP-4. This leads us to favor the hypothesis that the Pt activin B is binding to an activin receptor without activating the signal transduction cascade. Therefore, we would postulate that different domains of the mature activin protein are required for binding to and activation of the receptor. Throughout the TGF- β superfamily the phenylalanine residue changed to isoleucine in the Pt variant is highly con-

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Figure 10. Quantitation and specificity of Pt effect. Embryos were injected with RNA encoding mouse activin β A (β A), zebrafish activin β B (β B), or variants (Pt, CS) and analyzed after 32 hr. Relative frequency of the phenotypes observed was plotted against the amount and type of RNA injected. (A) Pt induces dose-dependent loss of axis. (B) CS variant suppresses the effect of wild-type activins on medaka embryos. (C,D) Coinjection of wild-type activin rescues T-protein expression in high Pt embryos. Embryos (~ 30 each) were injected with high amount of Pt (C) or Pt and a fivefold excess of activin β B, respectively (D). After 32 hr, expression of Brachyury protein was analyzed as described in Fig. 8. Note that epiboly proceeds to 70–90% in coinjected embryos (D, arrowhead) in comparison to high Pt embryo where only 30% epiboly is reached (C, arrowhead). Brachyury protein is detected at the level of the germ ring and in cells surrounding the oil droplets (arrows) in coinjected embryos (D), whereas no staining can be detected in the high Pt embryo (C). Further increase in wild-type RNA injection prevented the resumption of epiboly (E). CS variant suppresses the effect of Pt variant on medaka embryos. The data in the graphs are representative of three independent experiments.

served, with one exception. The only exception, MIS, inhibiting the formation of the Müllerian duct, contains a leucine residue at this same position. This region of the factor could itself be involved in the activation of the receptor or it could be required for the coupling of binding and activation functions. The first finger domain following the modified residue is thought to be involved in ligand–receptor interaction (Schlunegger and Grütter 1992). It may be required for specific binding of each ligand to its corresponding receptor, whereas another

“activation domain” is used universally. More detailed analysis of the variant–receptor interactions will help to clarify this point.

We have used CS and Pt variants to probe the role of activin during early embryogenesis. The injection of Pt RNA prevents mesoderm and axis formation in a dose-dependent manner. Taken together, our results argue strongly for the involvement of activin in those processes. Two control experiments make it unlikely that the effects observed are artifacts. First, the injection even

of highest amounts (120 pg) of the inactive CS variant does not obviously affect the embryonic development. Second, the effect of the Pt variant can be rescued by titrating it out with the CS variant. Different amounts of the Pt variant lead to the development of embryos with quite distinct phenotypes. The formation of caps peaks at highest Pt concentrations. The cap with structure is predominantly found at intermediate Pt concentrations but not or to a much lower extent at higher or lower concentrations. Embryos with reduced axial structures are most abundant after injection of the lowest amounts of Pt. The observation of graded effects after injection of the Pt variant is in good agreement with the idea of an activin activity gradient (Green et al. 1992). However, we find no evidence of asymmetric repartition of activin in the fish embryo by immunostaining. Because the Pt variant does not enable us to distinguish between activin B and activin A, it is possible that one form of activin (A, B, or AB) is present maternally and in a graded fashion. Alternatively, activin might be uniformly distributed but asymmetrically activated. Further experiments are needed to answer these questions.

It has been suggested that in *Xenopus* the zygotically transcribed activin β B might be involved in mesoderm induction (Thomsen et al. 1990). On the other hand, we and others find evidence for the presence of maternal activin in frogs (Asashima et al. 1991) and fish (Ge et al. 1993a; J. Wittbrodt and F. Rosa, this paper). CS RNA injection, even in excess does not perturb medaka development. Therefore, translation (and zygotic expression) of activin β B or β A is not required for mesoderm induction and axis formation in fish. Comparison of the effects of Pt and CS RNA clearly demonstrates that only maternal activin can be involved in these processes. Other factors have been implicated in those processes, including Vg1 and BMP-4, which are also present maternally (Weeks and Melton 1987; Köster et al. 1991; Dale et al. 1992; Jones et al. 1992). It will be interesting to use a similar approach to understand the respective involvement of those TGF- β -related molecules.

Inhibition of activin signaling in *Xenopus* has been postulated as a prerequisite for neural induction in *Xenopus* (Hemmati-Brivanlou and Melton 1992). High Pt injections led to high N-CAM levels in prospective ectoderm of medaka embryos. Our results are therefore in agreement with the enhanced N-CAM expression in animal caps from *Xenopus* embryos injected with XAR1-truncated activin receptor. However, in our hands, about half of high Pt embryos express no detectable N-CAM levels. Clearly, animal cap cells from Pt-injected embryos behave differently, depending whether they are maintained in the whole embryo environment or dissected away and cultured in isolation. One possible explanation is that animal caps cells from Pt-injected embryos receive additional signals that prevent them from becoming neuroectoderm. Alternatively, blocking activin activity might just reinforce a natural predisposition of ectoderm to become neuralized as established previously in *Urodeles* (Holtfreter 1948). Further experiments are needed to clarify this point.

In terms of evolution, the mechanism for the formation of mesoderm and the body axis utilized by lower vertebrates as well as the factors involved in this process appear to be highly conserved. The dominant-negative variants of activin introduced in this paper might provide the tools to address these questions and should be useful to elucidate the role of other members of the TGF- β family in vivo.

Materials and methods

PCR amplification and cloning of PCR products

We used modified degenerate oligonucleotides described previously (Thomsen et al. 1990). The amino-terminal primer is 5'-GGI (CT)TI GA(AG) TG(CT) GA(CT) GG-3'; the carboxy-terminal primer is 5'-ICC (AG)CA (CT)TC (CT)TC IAC IAT-3'. PCR amplification was performed on 10 ng of medaka and zebrafish genomic DNA. The 50- μ l reactions consisted of 1 $\times$ buffer, 0.2 mM each of dNTP, 4 μ M each primer, and 2 units of *Taq* DNA polymerase (Cetus). Reactions were cycled 35 times (1 min each at 92°C, 43°C, and 72°C) in a Peltier driven thermocycler (Wittbrodt and Erhardt 1989). The annealing temperature was increased through cycles 1–4 from 37°C to 43°C. After amplification, the reactions were run on a 3 + 1% NuSieve/agarose gel in 1 $\times$ TAE. The 350-bp product was cloned into the PCR-1000 vector (Invitrogen).

Cloning of full-length activin β B cDNA, expression in mammalian cells, and assays

The zebrafish activin β B clone was isolated from a cDNA library established from 20- to 28-hr-old zebrafish embryos and kindly provided by R. Riggleman and K. Helde (University of Utah, Salt Lake City) with the medaka activin β B probe. A 3.02-kb cDNA was sequenced in both directions following exonuclease III-nested deletions using the Sequenase kit (U.S. Biochemical). The complete sequence has been submitted to the EMBL data base (accession no. X76051). A 1260-bp *Bsp*HI–*Nsi*I fragment containing the entire coding region was subcloned into the *Eco*RI and *Pst*I sites of the pCMV5 vector (Andersson et al. 1989) to generate the plasmids pC β B (wild type), pC β B–CS (CS variant) and pC β B–Pt (Pt variant). COS cells were transfected by the DEAE–dextran method (Sambrook et al. 1989). 293 Cells were stably transfected with activin–pCMV constructs and a plasmid carrying neomycin resistance (pNEO β Gal, Stratagene) under G418 selection as described (Chen and Okayama 1987). Serum-free DMEM medium was conditioned for 60 hr and then assayed on stage 8 *Xenopus* animal caps at a 1:4 dilution in 1 $\times$ MMR (Gurdon et al. 1985; Smith 1987). SAOS cells treated with the appropriate supernatant for 2 days were processed for alkaline phosphatase in quadruplicates as described (Gray et al. 1987).

Site-directed mutagenesis, plasmid constructs, and in vitro transcriptions

Site-directed mutagenesis was performed on activin β B cDNA in pBluescript using the oligonucleotides 5'-CGC ATC CAT CAT TCA GGC TTG GAG-3' (CS-variant) and 5'-TCG ACA TCC GGC TCA T-3' (Pt-variant) according to Kunkel (1985). A 1260-bp *Bsp*HI–*Nsi*I fragment (see above) was ligated into the *Bgl*III-linearized pSP64T (Krieg and Melton 1984). Mouse ovary activin β A cDNA was obtained by RT–PCR and subcloned into pSP64T. Capped RNA was prepared as described after linearization of the plasmids with *Xba*I (Krieg and Melton 1984).

RNA injection into two-cell embryos

About 25 fg to 60 pg of mRNA was injected into one or two blastomeres at the two-cell stage. For injections, embryos were kept in 1× Yamamoto ringer (Yamamoto 1975) at 18°C, fixed in an agar well. RNA was diluted in 1× Yamamoto ringer containing 0.1% phenol red and 20 ng/μl of stage 15 poly(A)⁺ RNA. Injections were performed using glass capillary needles (3–5 μm in diam.). Injected embryos were transferred to a 27°C incubator. After 12 hr, the medium was changed to embryo-rearing medium containing 1% NaCl, 0.03% KCl, 0.04% CaCl₂, 0.16% MgSO₄, and 0.0001% methylene blue.

Injections of capped BMP-4 RNA into two-cell *Xenopus* embryos and measurement of *Xhox3* levels were performed exactly as described (Dale et al. 1992).

Oocyte injections and analysis of secreted proteins

Xenopus oocytes were prepared and injected with 10–30 ng of RNA as described (Krieg and Melton 1984). Injected oocytes (10) were pulse-labeled in 50 μl of OR2 medium with 30 μCi of [³⁵S]methionine for 2 hr. Oocytes were incubated further in fresh OR2 overnight. Supernatants were analyzed on 12% SDS-PAGE and processed for autoradiography.

RNA analysis

Total RNA was extracted as described (Sargent et al. 1986) and analyzed on Northern blots or by RNase protection using standard procedures (Sambrook et al. 1989). Gels were analyzed and quantitated using a Molecular Dynamics PhosphorImager. Autoradiographs were scanned and quantitated using the Molecular Dynamics image analysis system.

Isolation and RT-PCR analysis of RNA

Total RNA (500 ng) was DNase treated, phenol extracted, precipitated, and reverse transcribed using an oligo(dT) primer and 200 units of Moloney murine leukemia virus reverse transcriptase (GIBCO-BRL). Resulting cDNAs were amplified by PCR with 5′ primer 5′-CGA CAC GGG GGC TTA-3′ and 3′ primer 5′-GTA GTA CAG CAT GGA CA-3′ specific for medaka activin βB. The 50-μl reactions consisted of 1× buffer, 0.2 mM each of dNTP, 1 μM each primer, and 0.1 unit of *Taq* DNA polymerase (P.H. Stehelin, Basel). The reactions were cycled 25 times (92°C for 1 min, 52°C for 1 min, 72°C for 1 min), including a hot start.

Antibodies and immunohistochemistry

The mAb MZ 15 (Zanetti et al. 1985) was used at a dilution of 10^{−3}. T-protein antiserum (Schulte-Merker et al. 1993) was diluted to 0.2 × 10^{−4}. Medaka N-CAM was stained with mAb D-3 (Schlosshauer 1989; Bastmeyer et al. 1990) at a dilution of 10^{−4}. The mAb E4 against human activin was obtained from Nigel Groome (NIMR, London, UK) and used at a dilution of 10^{−3}. Embryos were fixed for >6 hr with 4% paraformaldehyde in 0.1 M phosphate buffer at pH 7.4 dechorionated and stained as described by Schulte-Merker et al. (1993).

Histology

After overnight fixation at ambient temperature in 4% paraformaldehyde in 1× PBS, medaka embryos were manually dechorionated, dehydrated, and processed through standard histological methods, including hematoxylin–eosin staining.

Dissection of medaka animal caps

Mid-blastula-stage medaka embryos (stage 10–11) were incubated for 30 min in hatching enzyme prepared according to Yamamoto (1975) and dechorionated manually. The animal-most 20% of the blastula was dissected off in L15 medium (GIBCO-BRL) using Tungsten needles. Single animal caps were transferred into agar-coated single wells of a 96-well plate and incubated in L15 medium at 28°C until sister siblings reached the 12-somite stage.

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