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Active complex formation of type I and type II activin and TGFβ receptors in vivo as studied by overexpression in zebrafish embryos

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Abstract

We have investigated the involvement of activin receptors and TGFβ type I receptor in zebrafish development. Overexpression of either full-length or a truncated form of mouse ActR-IIA interferes with the development. Different splice variants of mouse ActR-IIB have distinct effects; ActR-IIB4 induces abnormal embryos, whereas ActR-IIB2 does not. Activin and TGFβ type I receptors can induce axis duplications. Co-expression of ActR-IA or ActR-IB with the type II activin receptors results in a synergistic increase of the frequency of axis duplication. Moreover, ActR-IIB2 is synergistic with ActR-IA and ActR-IB, demonstrating that ActR-IIB2 can interact with the zebrafish ligand. Overexpression of TGFβR-I with ActR-IIA or ActR-IIB4 results in a synergistic increase in frequency of abnormal embryos, whereas in combination with ActR-IIB2 no such increase occurs.

Keywords: Zebrafish; Activin receptors; Axis formation

1. Introduction

The TGFβ superfamily of growth factors is a diverse group of signaling molecules which are involved in the differentiation and physiological activity of a wide range of cell types. Members of this superfamily and their cognate receptors have been implicated to be involved in mesoderm induction and axes formation/ patterning in the vertebrate embryo. The role of activin as well as bone morphogenetic protein-4 (BMP-4) and Vg1 (a BMP related protein) in early vertebrate development has been extensively studied in amphibians (Asashima et al., 1990; Smith et al., 1990; Thomsen et al., 1990, van den Eijnden-van Raaij et al., 1990; Koster et al., 1991; Dale et al., 1992; Jones et al., 1992; Dale et al., 1993; Smith, 1993; Schulte-Merker et al., 1994a). Activin and TGFβ bind to receptor heterodimeric complexes composed of type I and type II serine/threonine kinase receptors (for recent reviews see Mathews, 1994; ten Dijke et al., 1994a). The ligands initially bind their type II receptors, which subsequently associate with type I receptors to form an active signaling complex by activation of the serine/threonine kinase contained in the intracellular domain of the receptors. This mechanism has been described in detail for TGFβ and its receptors and postulates that the type I receptor requires a type II receptor to bind the specific ligand in order to activate the downstream signal transduction pathways (Wrana et al., 1994). Consequently, ligand specificity of type II receptors can be revealed relatively easily whereas the binding characteristics of a type I receptor can only be disclosed in the presence of the correct type II receptor. Thus far only one

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TGFβ type II receptor gene (TGFβR-II) has been identified (Lin et al., 1992), whereas for activin, two type II receptor genes are known; ActR-IIA (Mathews and Vale, 1991) and ActR-IIB (Attisano et al., 1992), of which the latter encodes four different splice variants in the mouse. Six different type I receptor genes (nomenclature reviewed by Mathews, 1994) have been cloned and their ligand specificities have been characterized. TGFβR-I (ALK-5, R4, Franzén et al., 1993; He et al., 1993) is involved in both binding and signaling of TGFβ in a complex with TGFβR-II (Franzén et al., 1993). ActR-IA, (TskL7, ALK-2, R1, ActRI; Attisano et al., 1993; Ebner et al., 1993; He et al., 1993; ten Dijke et al., 1993) and ActR-IB (ALK-4, R2; ten Dijke et al., 1993; He et al., 1993; Carcamo, 1994; ten Dijke et al., 1994b) have been identified as activin type I receptors, since these receptors bind activin in the presence of activin type II receptors. In addition ActR-IA is a type I receptor for osteogenic protein-1 (OP-1), whereas ALK-3 and BMPR-IB, also known as ALK-6, are type I receptors for BMP-4 and OP-1 (ten Dijke et al., 1994c).

In *Xenopus* it has been concluded from experiments in which full-length or truncated type II activin receptors were overexpressed that activin receptor signaling pathways may be involved in vivo in the process of mesoderm induction and axis formation (Kondo et al., 1991; Mathews et al., 1992; Hemmati-Brivanlou et al., 1992; Hemmati-Brivanlou and Melton, 1992; Nishimatsu et al., 1995). Such truncated activin type II receptors can bind ligand but lack the intracellular kinase domain which is involved in signal transduction and as such are supposed to function as dominant negative variants.

We have systematically studied the involvement of activin receptors and TGFβ receptors in early embryonic zebrafish development and explored the zebrafish as a vertebrate model to determine the potential complex formation of type I and type II receptors in vivo. Activin has been shown to induce in vitro the expression of several primary response zebrafish genes which are the earliest markers for mesoderm induction. Upon treatment of zebrafish animal caps with activin the expression of *no tail*, the zebrafish homologue of mouse *BrachyuryT* (Schulte-Merker et al., 1992, 1994b) and *snail* (Hammerschmidt and Nusslein-Volhard, 1993) are induced. Treatment of dissociated zebrafish blastula cells with activin results in increased expression of *axial* (Strähle et al., 1993) and *goosecoid* (unpublished observations, J. Joore). The expression patterns of these genes in the zebrafish embryo strongly suggest that the gene products are involved in mesoderm formation in the zebrafish embryo and that the expression of these genes can be regulated upon binding of activin to zebrafish serine/threonine kinase receptors.

In the present study we demonstrate that truncated and full-length activin and TGFβ receptors when overexpressed interfere with normal zebrafish development. The disturbance of zebrafish development upon overexpression of truncated mouse ActR-IIA is specific, since the abnormalities thus induced are rescued upon co-injection of full-length ActR-IIA. Overexpression of different splice variants of full-length mouse ActR-IIB results in different effects in zebrafish embryos; ActR-IIB2 does not interfere with normal development whereas ActR-IIB4 does. The zebrafish embryo was further exploited for co-expression of different combinations of type I and activin type II receptors to assess the in vivo complex formation between activin type II receptors and type I receptors. From our data we conclude that these receptors are involved in axis formation and that the binding characteristics of the endogenous zebrafish ligand(s) which interact with the activin receptors are different from the known binding specificity of activin for these receptors.

2. Results

2.1. Overexpression of full-length and truncated type II activin receptors disturbs zebrafish development

In order to determine the relevance of activin type II receptors in zebrafish development, we overexpressed full-length and truncated forms of these receptors in early zebrafish embryos and assayed the effects on embryonic development. Here we show that ectopic expression of truncated mouse ActR-IIA which lacks the complete intracellular kinase domain (see Section 4 for details on construct) interferes with the normal development of zebrafish embryos. RNA encoding the truncated receptor was injected at the interphase between yolk and cytoplasm of 1-cell to 4-cell stage zebrafish embryos. To assess correct expression, the receptor sequences were epitope-tagged (Field et al., 1988) and whole mount receptor immunostaining was performed. The exogenous receptors were expressed correctly in the cell membrane and could be distributed evenly throughout the embryo as shown in Fig. 1C. However, most often the expression pattern was somewhat mosaic (Fig. 1 A,B) which is in agreement with the expression patterns observed upon RNA injections in *Xenopus* embryos (Amaya et al., 1993). Probably, the variable expression is due to the slow diffusion of the RNA from the injection site and the rapid partitioning of the cytoplasm during cleavage. The extent of mosaic expression in the zebrafish embryos was similar for injections performed at the 1-cell up to the 4-cell stage independently of whether RNA was introduced in the yolk–cytoplasm interface or in the cytoplasm directly.

During the gastrulation no gross morphological differences were observed between uninjected and control injected embryos and epiboly proceeded at normal rate. However, when we analyzed the embryos 30 h post fertilization, the injected embryos were clearly affected in comparison to uninjected or control injected embryos (Fig. 1D). We have chosen to analyze embryos at 30 h

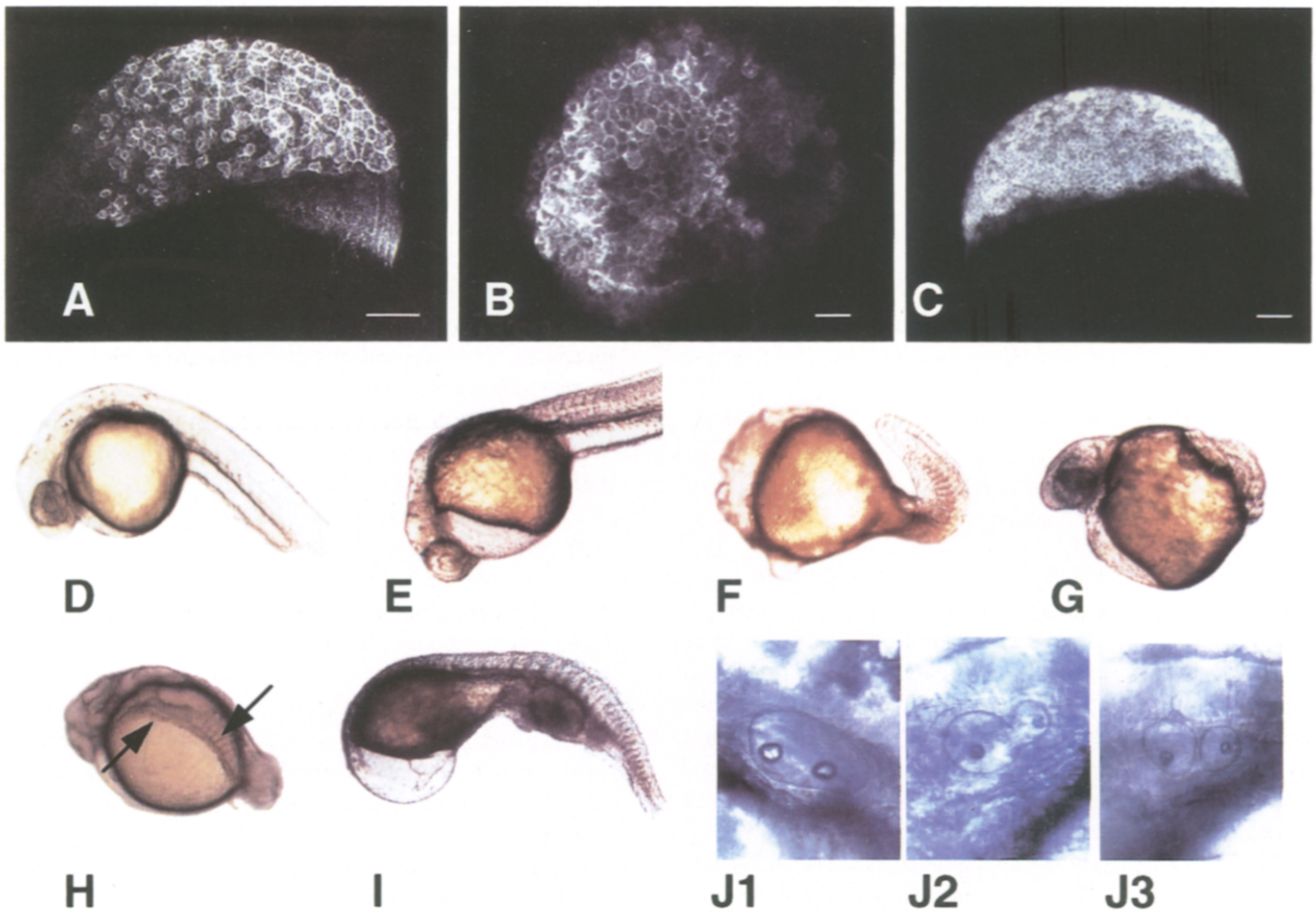

Fig. 1. Overexpression of truncated ActR-IIA or full-length ActR-IIA disturbs normal zebrafish development. Zebrafish embryos were injected at the 1-cell to 4-cell stage with water (D), or with in vitro capped RNA encoding either truncated ActR-IIA (A–C and E–H) or wild-type ActR-IIA (I–J). Injected embryos were fixed at 50–60% epiboly and processed to immunohistochemistry (see Section 4) with the anti-tag antibody (12CA5) to show correct expression of the injected RNA. After incubation with the secondary fluorescently labeled antibody, the embryos were analyzed by confocal laser scanning microscopy. Mosaic expression of truncated ActR-IIA (A,B). Homogenous expression of truncated ActR-IIA (C). Embryos were injected as mentioned above and subsequently allowed to develop and analyzed at 30 h of development. Control, water-injected embryos, developed normally (D). Embryo displaying mild abnormalities with the eyes almost fused and the anterior head structures reduced (E). Embryo lacking all head structures (F). Embryo characterized by a severely truncated tail (G). Most severely affected embryo, no head or tail structures. Note the presence of some square somites and notochord (arrows) (H). Completely ventralized embryo due to overexpression of full-length ActR-IIA (I). Detail of the otic vesicle of wild type embryo (J1) and defects observed upon overexpression of full-length ActR-IIA; duplication of the otic vesicle (J2,3). For (A) and (C), a lateral view, animal pole up; in (B) lateral view, animal pole to the left top; in (D–I) a lateral view with anterior to the left. In (J) only the otic vesicle is shown in a lateral view.

post fertilization since by that time a complete body plan has been established, tail straightening has been completed and blood flow through the vascular system takes place. Overexpression of truncated ActR-IIA caused a range of distinct malformations (see Table 1). Either the anterior structures were affected slightly, e.g. one or both eyes were missing, the eyes were fused, the telencephalon was truncated (Fig. 1E) or, at a tenfold lower frequency, all the anterior structures including rhombomere 5 and the otic vesicle were missing (Fig. 1F). Minor posterior defects such as a bent tail or partial truncation of the tail were observed at a threefold higher frequency than in the control injected embryos. In 6% of the embryos, a complete truncation of the tail was observed (Fig. 1G) and in 22% of the injected embryos, both anterior and posterior structures were missing (Fig. 1H). Even in the most severely affected embryos, there were always dorsal mesodermal structures as (square) somites or short stretches of notochord (Fig. 1H).

Overexpression of full-length ActR-IIA results in a different pattern of abnormalities compared to those observed upon injection of RNA encoding truncated ActR-IIA. The most severe phenotype was rarely observed. Typically, embryos injected with RNA encoding full-length ActR-IIA displayed malformations of the early ear structures at a high frequency of 44%. Interestingly, such abnormalities never occurred upon overexpression of truncated ActR-IIA. Either the presence of only one instead of two otoliths per otic vesicle was observed or multiplication of the otic vesicle and/or the otoliths (Fig.

Table 1
Axial defects in embryos injected with truncated or full-length Act IIA

	Control	Truncated ActR- IIA	ActR- IIA
No. of embryos injected	56	183	106
Normal embryos (Fig. 1D) (%)	96	28	58
Mild anterior defects (Fig. 1E) (%)	0	28[a]	27[b]
Anterior structures including otic vesicle missing (Fig. 1F) (%)	0	2	4
Minor posterior defects (%)	4	14	5
No tail, tail severely truncated (Fig. 1G) (%)	0	6	1
Severely affected anterior and posterior (Fig. 1H) (%)	0	22	5

One nanogram of RNA encoding either truncated or full-length ActR-IIA was injected at the 1–4 cell stage (see Section 4) and the embryos were analyzed at 30 h post fertilization.
[a]No defects in otic vesicles.
[b]19 out of 29 embryos with defects in otic vesicle.

1J). Furthermore, completely ventralized embryos were observed without any anterior structures, consisting of an enlarged pericardium with a truncated but beating heart and a short tail with square somites (Fig. 1I). In contrast to what has been observed upon overexpression of full-length ActR-IIA in *Xenopus* embryos, secondary axes were not induced.

Injection of increasing concentrations of RNA encoding truncated ActR-IIA results in an increasing frequency of abnormal embryos reaching 100% (Fig. 2A). Injection of increasing doses of RNA encoding full-length ActR-IIA resulted in increasing percentages of abnormal development of zebrafish embryos, although 45% was the highest percentage that could be achieved (Fig. 2A). To establish specificity of the effects observed upon overexpression of truncated ActR-IIA, we performed a rescue experiment with full-length ActR-IIA. To rescue the effect of the truncated receptor, a constant amount of RNA encoding truncated ActR-IIA was co-injected with increasing doses of full-length ActR-IIA RNA which at a concentration of 600 ng/μl results in a complete rescue (Fig. 2B). At concentrations of full-length ActR-IIA RNA of 600 ng/μl and higher, abnormal ear structures were observed at high frequency which is, as previously described, an abnormality specific for full-length ActR-IIA phenotype. These data show that the effects of overexpressed truncated ActR-IIA on zebrafish development can be rescued by co-expression of the full-length receptor, indicating that the observed malformations are not due to physical disruption of the embryos or toxicity of the RNA, but to specific interference of the overexpressed receptor (variants) with endogenous signaling pathways. Only at an intricately balanced co-expression of truncated and full-length ActR-IIA does normal development take place since both the impaired function as well as enhanced activity of this receptor seem to affect zebrafish development.

Subsequently, we investigated the effects of overexpression of ActR-IIB; four different splice variants have been described in the mouse. These splice variants exhibit

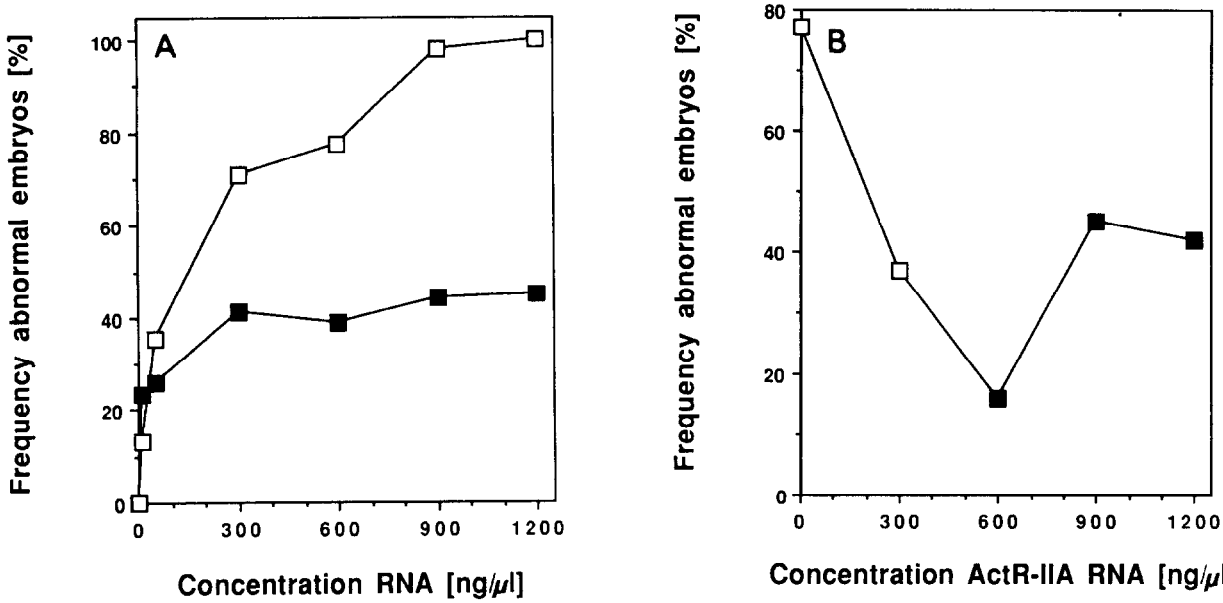

Fig. 2. Dose dependent induction of abnormal embryonic phenotypes upon overexpression of truncated and wild-type ActR-IIA and rescue of the truncated ActR-IIA phenotype by full-length ActR-IIA. (A) The frequency of defects at 30 h of development upon injection of increasing concentrations of RNA encoding truncated ActR-IIA (open squares) or wild-type ActR-IIA (closed squares). (B) Rescue of defects induced due to injection of a constant amount of RNA encoding truncated ActR-IIA (600 ng/μl) by co-injection of increasing concentrations of RNA encoding wild-type ActR-IIA, expressed as the frequency of abnormal embryos. The line is composed of two different symbols, with open squares for phenotypes induced by truncated ActR-IIA and closed squares when malformations specific for full-length ActR-IIA, like aberrant otic vesicles, are observed at high frequency. For each point, at least 30 embryos were injected and analyzed.

different affinities for activin ranging from high for ActR-IIB1 and −2 (K_d 100 pM) to low for ActR-IIB3 and −4 (K_d 380 pM) (Attisano et al., 1992). In zebrafish embryos, we overexpressed the two variants which are identical in their intracellular domain and both lack the exon encoding 24 amino acids intracellularly but differ in their extracellular domains; ActRII-B2 contains 8 amino acids which lack in the extracellular domain of ActR-IIB4. At the carboxy terminus, the cDNAs were extended with the epitope which is recognized by the monoclonal antibody KT3 (MacArthur and Walter, 1984) such that correct expression could be assayed upon RNA injection identical to the data obtained for ActR-IIA both for ActR-IIB2 and ActR-IIB4 (data not shown; see Fig 1A–C). Overexpression of both full-length and truncated ActR-IIB2 in zebrafish embryos did not affect development, even though RNA was injected at very high concentrations (up to 3600 ng/μl). In contrast, both the full-length and the truncated form of the shorter splice variant ActR-IIB4 interfered very efficiently with signaling of endogenous receptors resulting in a high frequency of abnormal embryos. The abnormalities observed were very similar to those described for overexpression of full-length and truncated ActR-IIA, respectively; again no ectopic axes were observed.

2.2. *Overexpression of type I serine/threonine kinase receptors*

ActR-IA and ActR-IB have been shown to interact with type II activin receptors in binding of activin and subsequent signal transduction (Ebner et al., 1993; Carcamo et al., 1994). Overexpression of either full-length mouse ActR-IA or full-length mouse ActR-IB in zebrafish embryos results in axis duplication (Fig. 3A–D). Axis bifurcation was observed to different extents and always started from the trunk or just anterior to the tail and was propagated anteriorward, resulting in bifurcated embryos with one common tail.

TGFβR-I has been identified as a TGFβ type I receptor, which binds TGFβ upon co-expression with the type II TGFβ receptor and mediates the TGFβ signal (Franzén et al., 1993; Bassing et al., 1994; Wrana et al., 1994). Overexpression of the human TGFβ type I receptor disturbs normal zebrafish development. In analogy to the effects observed for ActR-IA and ActR-IB, TGFβR-I can also induce axis duplication (data not shown; see Table 2). These data indicate that the human TGFβ type I receptor can interact with an endogenous zebrafish ligand as well as with a zebrafish type II receptor.

2.3. *Co-injection of RNAs encoding type I and type II receptors*

At present not all ligands for the serine/threonine kinase receptors have been identified or are not available in sufficient quantities to perform binding and cross-linking studies in order to determine the potential complex formation between type II activin receptors and known type I receptors. Therefore, we co-expressed these receptors in zebrafish embryos to assess for the synergis-

Table 2

Co-injection of type II activin receptors and type I receptors: frequency of severely affected embryos upon overexpression of type I and type II receptors separately or in combination

RNA injected (ng/μl)	n[a]	Abnormal embryos (%)		
A				
ActR-IA (50)	33	33		
ActR-IB (100)	45	18		
TGFβR-I (600)	127	13		
ActR-IIA (900)	25	8		
ActR-IIB2 (600)	72	0		
ActR-IIB4 (300)	58	19		
RNA injected	n[a]	Abnormal embryos (%)	Calculated	
			Abnormal embryos (%)[b]	x-fold[c]
B				
ActR-IA + ActR-IIA	64	65	41	1.6
ActR-IA + ActR-IIB2	82	64	33	2.0
ActR-IA + ActR-IIB4	66	89	52	1.7
C				
ActR-IB + ActR-IIA	99	42	26	1.6
ActR-IB + ActR-IIB2	52	40	18	2.2
ActR-IB + ActR-IIB4	37	62	37	1.7
D				
TGFβR-I + ActR-IIA	42	60	21	2.9
TGFβR-I + ActR-IIB2	97	10	13	0.8
TGFβR-I + ActR-IIB4	40	58	32	1.8

(A) Different concentrations of RNA encoding the different receptors were chosen such that at most 1 out of 3 embryos was severely affected. Only embryos severely affected were taken into account (see Section 2). An embryo is considered severely affected when either complete anterior or posterior structures are missing, or when axis duplications are present. Embryos in which, e.g. only a duplication of the otic vesicle was observed were not considered as severe, which explains the discrepancy of 8% abnormal embryos due to injection of 900 ng/μl RNA encoding ActR-IIA as given in this table, whereas in the graph of Fig. 2A a total percentage of 52% abnormal embryos is given. (B) Overexpression of ActR-IA in combination with the different type II activin receptors. (C) Overexpression of ActR-IB in combination with the different type II activin receptors. (D) Overexpression of TGFβR-I in combination with type II activin receptors. The results of a typical experiment are given.

[a] n, number of embryos injected.

[b] Frequency of abnormal embryos as expected from the data given in **A**.

[b] Relative comparison of actual frequency of abnormal embryos upon co-expression of type I and type II receptors and frequency as calculated from the data in Table 2A. A relative comparison which results in a larger number than 1, indicates that the co-expressed receptors result in a synergistic effect.

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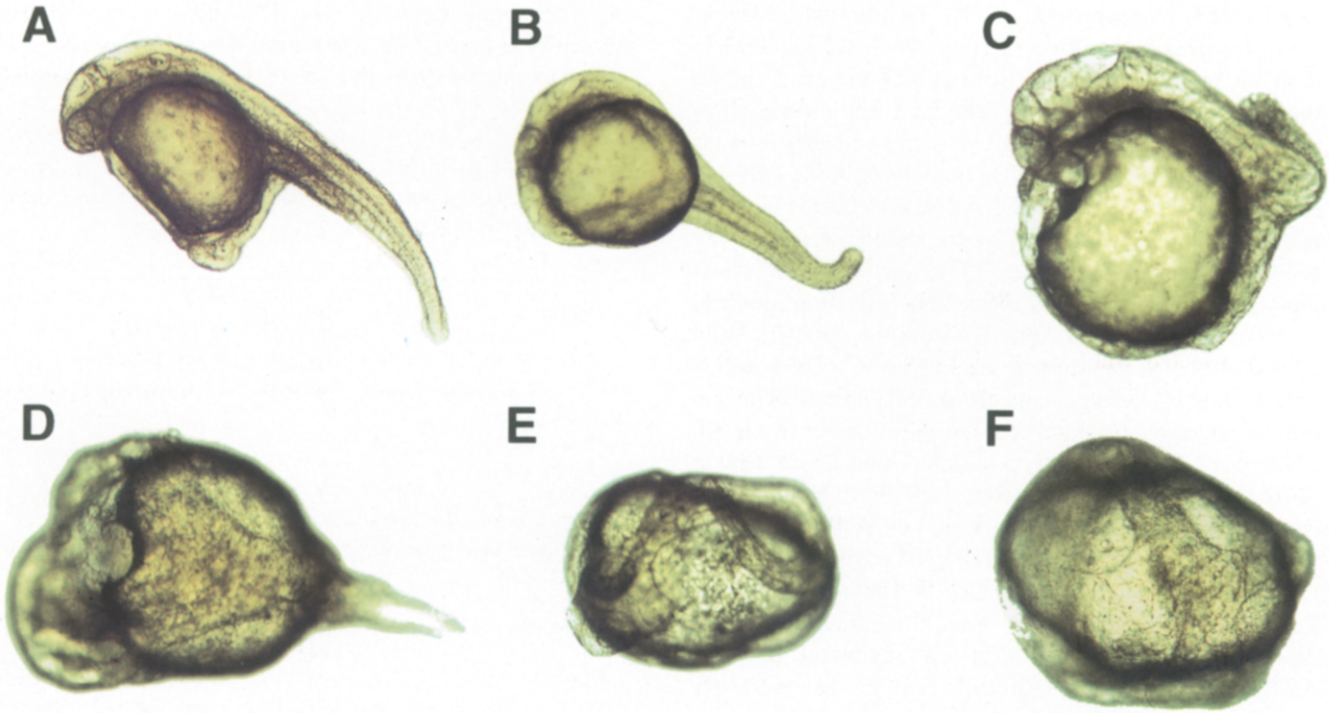

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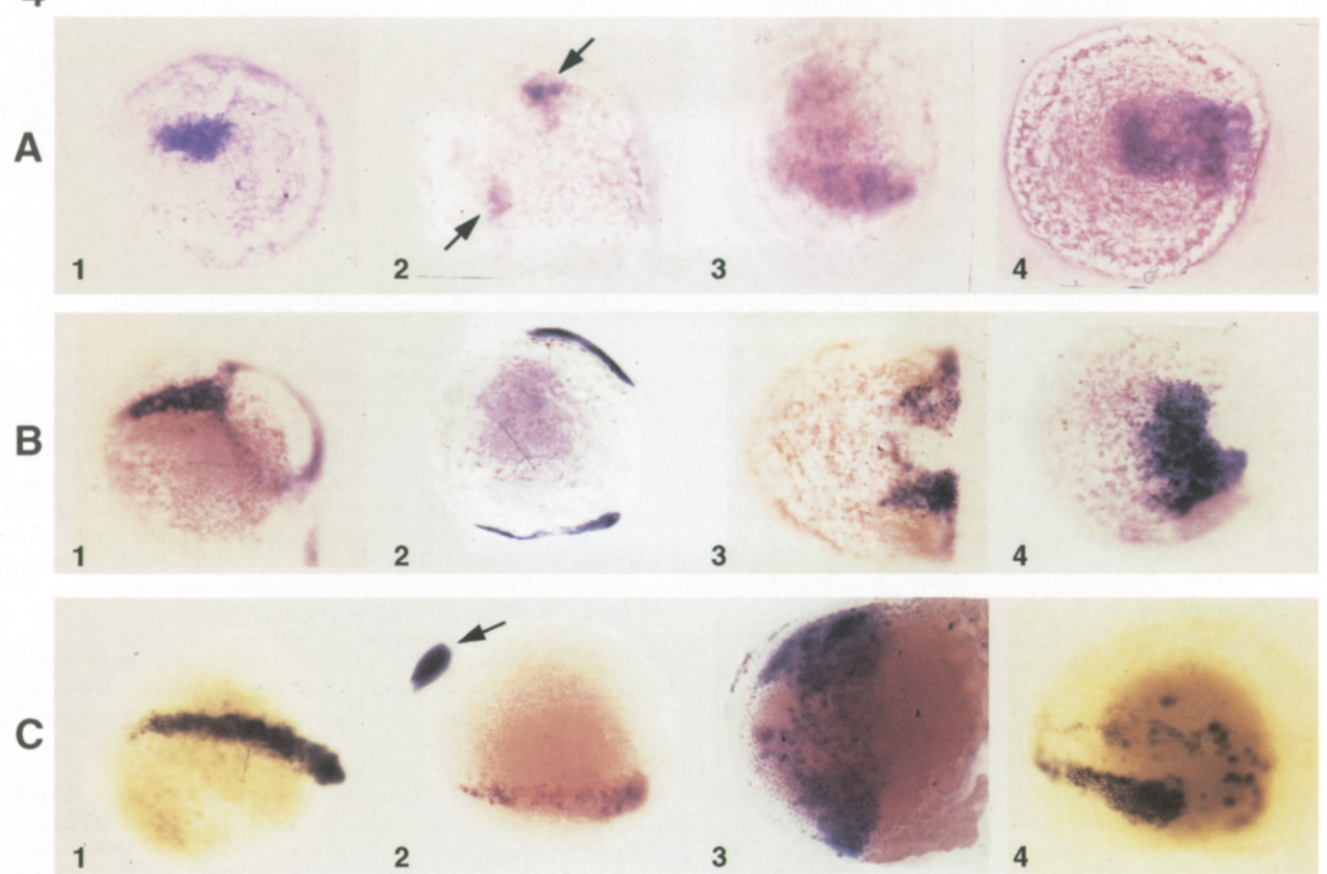

tic disturbance of normal development as an indication of signaling complex formation. RNAs encoding type I and/or type II receptors were co-injected in zebrafish zygotes to determine which combination of type I and type II receptors would result in the formation of an active signaling receptor complex in this system. The concentration of RNA for a single receptor was chosen such that at most one out of three injected embryos was severely affected (Table 2). This was done in order to score the maximum increase in phenotypes occurring as a consequence of co-injection of both receptors type I and II. In these experiments only the frequency of severely affected embryos was determined. An embryo is defined as severely affected when dramatic effects on either anteroposterior patterning were observed such as the absence of the tail, the head or both head and tail or when axis duplications were observed (Fig. 3). When multiple axes are formed the phenotype is very complex and difficult to assess as is shown in Fig. 3E,F. Therefore we did not score the frequency of embryos with axis duplications separately. In Table 2B–D the frequency of abnormal embryos measured upon co-injection of type I and type II receptors is given in the third column. Furthermore, the percentage of affected embryos obtained by adding up the percentages brought about by each of the receptors alone (Table 2A) is depicted as well as the comparison between the actual and calculated percentages. From the data presented in Table 2B and C we conclude that both ActR-IA and ActR-IB form active heteromeric complexes with all three type II receptors tested, because co-injection of the receptors results in a 1.6–2.2-fold higher incidence of severe phenotypes than expected from the addition of percentages determined upon separate injection of the receptors (Table 2A). Clearly, ActR-IIB2 which alone did not affect development can form active complexes with ActR-IA and ActR-IB resulting in a synergistic effect on development. This indicates that ActR-IIB2 may interact with an endogenous zebrafish ligand but does not form ternary complexes with endogenous type I receptors since even high concentrations of injected ActR-IIB2 mRNA do not affect zebrafish development. We thus conclude that ActR-IIB2 can interact with a zebrafish ligand which again shows that the effect of overexpressed receptors is not due to absorbance of endogenous growth factor, but rather due to the specific active complex formation with endogenous receptors.

Co-injection experiments of TGFβR-I with ActR-IIA or ActR-IIB4, similarly cause a synergistic effect on zebrafish development (Table 2D). However, co-injection of TGFβR-I and ActR-IIB2 does not result in an increased effect, from which we conclude that ActR-IIB2 does not form complexes with TGFβR-I. Importantly, these data indicate that no ligand-independent complex formation and subsequent signaling takes place as a consequence of high expression levels of the overexpressed receptors.

2.4. Expression pattern of primary response genes goosecoid and no tail and of early mesodermal marker axial upon injection of type I receptors alone or with type II receptors.

Defects in embryos injected with RNAs encoding the different receptors were determined at 28–30 h after fertilization. To further investigate whether the injection of type I receptors alone or in combination with type II receptors affects axis formation, the expression pattern of several mesodermal markers, *no tail*, *goosecoid* and *axial* (Schulte-Merker et al., 1992; Stachel et al., 1993; Strähle et al., 1993) was analyzed during gastrulation. In agreement to what was found for the abnormal embryos shown in Fig. 3 the expression pattern of these markers is very pleiotropic in injected embryos. At 80% epiboly, the expression of *goosecoid* is restricted to the anterior shield, as is shown in Fig. 4A1 in which in situ hybridization with a *goosecoid* probe was performed on an uninjected embryo. At this stage *no tail* is expressed in cells in the germ ring and in the presumptive notochord (Fig. 4B1), whereas *axial* is expressed in a band of cells at the dorsal side of the embryo from the blastoderm margin to the

Fig. 3. Overexpression of type I receptors alone or in combination with type II receptors results in axis duplication as assessed at 30 h of development. (A) Embryo with one complete and one additional partial axis induced upon ectopic expression of ActR-IB, lateral view, anterior to the right. (B) Embryo injected with ActR-IB and ActR-IIA with two almost complete axes in which the most anterior regions appear to be fused. (C) Embryo with two incomplete axes and a common truncated tail, injected with ActR-IB and ActR-IIB2, anterior to the left. (D) Dorsal view of axis bifurcation anterior to the tail, due to co-injection of ActR-IB and ActR-IIB2. (E,F) Embryos with multiple axes due to ectopic expression of ActR-IA and ActR-IIB4, dorsal view.

Fig. 4. Whole mount in situ hybridization at 80–90% epiboly of wild-type and injected embryos with (A) *goosecoid*, (B) *no tail* and (C) *axial* probes. (A1) Wild type *goosecoid* expression, (B1) wild type *no tail* expression and (C1) wild type *axial* expression. Examples of embryos with a clear second axis; (A2) *goosecoid* in embryos co-injected with ActR-IB and ActR-IIB2 (the two spots of expression are indicated with arrows), (B2) *no tail* expression in embryos co-injected with ActR-IA and ActRIIB4 and (C2) *axial* expression upon injection of ActR-IB (of the second axis only the broader base is visible at the arrow). Two broader bands of expression much wider than normal are shown in (A3) for *goosecoid* upon overexpression of ActR-IB and ActR-IIB4, (B3) for *no tail* in ActR-IB and ActR-IIA injected embryos and (C3) for *axial* in embryos overexpressing ActR-IA and ActR-IIA. An expression of the markers which is wider than normal or with staining additional to the normal pattern is shown in (A4) for *goosecoid* upon TGFβR-I injection, (B4) for *no tail* upon overexpression of ActR-IB and (C4) for axial in embryos injected with ActR-IB and ActR-IIB4. Similar expression patterns were observed for each of the injections of type I receptors alone or in combination with the type II receptors and the results given in this figure should be considered as representative examples of expression patterns rather than as typical examples due to injection of the different RNAs (see Section 2).

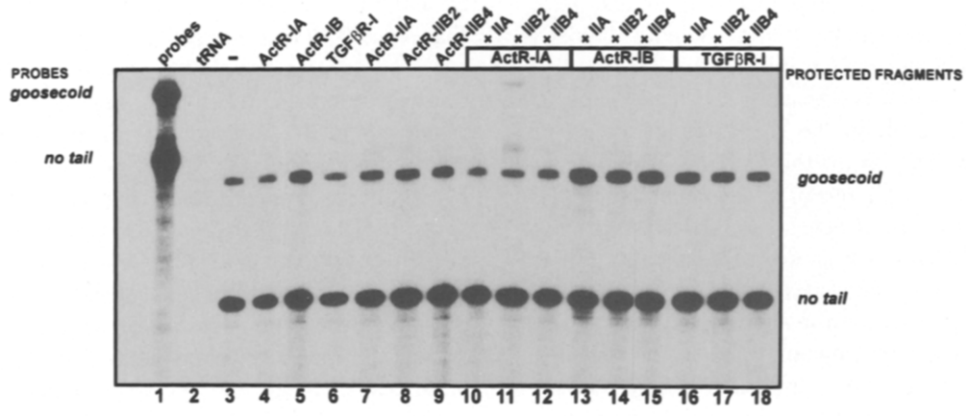

Fig. 5. Expression of *goosecoid* and *no tail* as determined by RNase protection analysis in injected embryos. Total RNA was isolated at 80–90% epiboly of uninjected embryos (lane 3), embryos injected with ActR-IA (lane 4), ActR-IB (lane 5), TGFβR-I (lane 6), ActR-IIA (lane 7), ActR-IIB2 (lane 8), ActR-IIB4 (lane 9), ActR-IA and -IIA (lane 10), ActR-IA and -IIB2 (lane 11), ActR-IA and -IIB4 (lane 12), ActR-IB and -IIA (lane 13), ActR-IB and -IIB2 (lane 14), ActR-IB and -IIB4 (lane 15), TGFβR-I and ActR-IIA (lane 16), TGFβR-I and ActR-IIB2 (lane 17) and TGFβR-I and ActR-IIB4 (lane 18) and hybridized to *goosecoid* and *no tail* anti-sense probes (lane 1) and subjected to RNase treatment. The RNase resistant fragments were run on a sequencing gel and visualized by autoradiography. In lane 2 the control with tRNA which does not protect the probes against RNase digestion is shown. The positions of the probes and the protected fragments for *goosecoid* and *no tail* are indicated. A typical experiment is shown, the concentrations of the injected RNAs were as depicted in Table 2.

animal pole. This band is slightly broader at the margin and narrows down towards the animal pole (Fig. 4C1). Several examples of the expression patterns of these markers due to overexpression of full-length receptors are shown in Fig. 4. Examples in which two separate axes are identified upon in situ hybridization are shown in Fig. 4A2 for *goosecoid*, Fig. 4B2 for *no tail* and in Fig. 4C2 for *axial*. In Figs. 4A3, B3 and C3 two or more major bands of expression are visualized with the different probes. Overexpression of type I receptors with or without type II activin receptors could also result in a broader band of expression than normal at this stage of development (shown for *goosecoid* in Fig. 4A4 and *no tail* in Fig. 4B4) or in a more or less normal band of marker expression and additional expression in randomly distributed cells as is shown for *axial* in Fig. 4C4.

Table 3

Phospholmager data of RNase protection analysis on *goosecoid* and *no tail* expression

	gsc	*ntl*	*gsc/ntl*
Uninjected	53	27	2.0
ActR-IA	52	20	2.6
ActR-IB	123	41	3.0
TGFβR-I	50	21	2.4
ActR-IIA	74	31	2.4
ActR-IIB2	91	47	1.9
ActR-IIB4	90	53	1.7
ActR-IA + ActR-IIA	57	34	1.7
ActR-IA + ActR-IIB2	68	37	1.8
ActR-IA + ActR-IIB4	80	28	2.9
ActR-IB + ActR-IIA	233	33	7.1
ActR-IB + ActR-IIB2	179	38	4.7
ActR-IB + ActR-IIB4	220	28	7.9
TGFβR-I + ActR-IIA	124	40	3.1
TGFβR-I + ActR-IIB2	110	42	2.6
TGFβR-I + ActR-IIB4	95	35	2.7

Quantitative analysis of the RNase protection analysis data of Fig. 5 (see legend of Fig. 5 for details on the experimental procedure). The relative intensities of the radioactive bands was determined by PhospholImager analysis and expressed in arbitrary units. In the last column the ratio of the intensity of *goosecoid* expression and the *no tail* expression in the same sample was calculated. *gsc*, *goosecoid*; *ntl*, *no tail*; *gsc/ntl*, relative expression of *goosecoid* over *no tail* as calculated from the data upon analysis of the RNase protection data by PhospholImager.

Subsequently, we assayed the expression levels of *goosecoid* and *no tail* by RNase protection analysis. After injection, 10 embryos were randomly chosen and fixed at 80–90% epiboly for RNA isolation. The RNAs were hybridized to *goosecoid* and *no tail* probes and subjected to RNase treatment. The protected fragments were run on a gel and visualized by autoradiography (Fig. 5). The intensities of the bands were determined with the PhosphoImager and the data are expressed in arbitrary units and summarized in Table 3. From the ratio of *goosecoid* over *no tail* expression, it becomes clear that the expression of *goosecoid* is significantly increased upon overexpression of ActR-IB with each of the activin type II receptors (Table 3). This specific induction of *goosecoid* expression due to overexpression of these receptors in comparison to the expression of *goosecoid* in embryos injected with other receptor combinations suggests that activation of *goosecoid* and *no tail* requires the activation of specific signal transduction pathways.

3. Discussion

In this study we show that overexpression of both full-length and truncated activin type II receptors as well as full-length type I serine/threonine kinase receptors can interfere with normal zebrafish development. Furthermore, we exploited the zebrafish as an in vivo model system to assay differences in functional characteristics of splice variants of ActR-IIB and the potential complex formation between type I and type II receptors.

To validate our procedure for zebrafish embryo injections, we first showed that injection in the cytoplasm at the 1-cell stage results in similar expression patterns as assessed by whole mount antibody labeling, as injection at the interphase of yolk and cytoplasm at the 1-cell to 4-cell stage. The receptors are expressed correctly at the cell membrane but the expression pattern is mosaic. Overexpression of truncated ActR-IIA results in a severe disturbance of the antero-posterior axis, whereas the mesoderm induction is not inhibited completely as even the most severely affected embryos exhibit some mesodermal structures. Although some degree of consistency in abnormalities can be discerned, as we grouped them in several categories, the issue of reproducibility of abnormalities obtained is complicated by mosaicism. It could also be speculated that the promiscuity of the introduced receptors leads to their involvement in distinct signaling pathways. The effects due to overexpression of truncated ActR-IIA are completely rescued by wild-type ActR-IIA which shows that the effects observed are specific.

In the present study we show that the two splice variants of mouse ActR-IIB have distinct effects on zebrafish development; the shortest variant, ActR-IIB4, induces abnormal embryos, whereas ActR-IIB2 is expressed correctly but does not affect zebrafish development. From binding experiments in which these receptors are expressed in COS cells, it is known that the affinity of activin is 3.8-fold higher for ActR-IIB2 than for ActR-IIB4 (Attisano et al., 1992). To explain our data we initially speculated that the endogenous zebrafish ligand is an activin-like ligand with different binding characteristics such that it can only bind ActR-IIB4 with high affinity. However, upon co-injection of ActR-IIB2 with either ActR-IA or ActR-IB, a synergistic effect is observed, indicating that an endogenous ligand has the capacity to bind ActR-IIB2, but rather that no ternary complexes are formed between ActR-IIB2 and endogenous type I receptors. This possibility is further supported by the data obtained from the co-injection of ActR-IIB2 and TGFβR-I, which does not show a synergistic effect, indicating that the co-expression of these receptors does not result in the formation of a functional complex. We conclude that the eight variable amino acids in the extracellular region of ActR-IIB splice variants are probably involved in the specificity of complex formation of this type II receptor with type I receptors.

Overexpression of either full-length ActR-IIA or ActR-IIB in zebrafish embryos does not result in the induction of secondary body axes, which is in contrast to the data obtained in *Xenopus* where axis duplication due to overexpression of full-length ActR-IIA or ActR-IIB was observed (Kondo et al., 1991; Hemmati-Brivanlou et al., 1992). This discrepancy could be explained by differences in type I receptor expression patterns in the different organisms. An alternative explanation could be that the putative mesoderm inducing signal is uniformly distributed throughout the zebrafish blastoderm as is the case for the zebrafish homologue of *Xenopus* Vg-1 (Helde and Grunwald, 1993) and that mosaic expression of truncated ActR-IIA will always allow a region of the embryo to be free of truncated protein and therefore with availability of type I receptor to signal and induce dorsal axial structures.

The fact that overexpression of type I activin and TGFβ receptors interferes with normal development could reveal the mechanism by which these overexpressed receptors disturb normal signaling of the endogenous receptors. For activin and TGFβ type I receptors, it has been shown that the ligand cannot bind unless a type II receptor is present which would indicate that it is not merely absorbance of the ligand by the overexpressed receptors, but rather ternary complex formation with endogenous ligand(s) and receptor(s) and the consequent signal transduction which results in abnormal development. However, recent binding studies in tissue culture cells revealed that BMPs can bind type I receptors with high affinity in the absence of type II receptors (Koenig et al., 1994; Penton et al., 1994; Graff et al., 1994). These data do not support the above proposed mechanism of action of overexpressed receptors, although it is unclear how such in vitro data exactly extrapolate to the in vivo situation. Our proposed explanation is in accordance with the data recently obtained from *Drosophila* mutant analysis in which there is an absolute requirement for both the type II and type I receptors, *punt* and *thick venes*, in order for dpp signaling to take place (Letsou et al., 1995; Ruberte et al., 1995).

The TGFβ type I receptor (Franzén et al., 1993) was not expected to affect zebrafish development, since TGFβ does not induce mesoderm in the *Xenopus* animal cap assay. However, upon ectopic expression of the TGFβR-II, *Xenopus* blastula cells become responsive to added TGFβ indicating that the cells express an endogenous type I receptor which can bind TGFβ and mediate its signal (Bhushan et al., 1994). Probably, the TGFβR-I does not only bind and signal in response to TGFβ, but also has a broader ligand specificity. Such cross-reactivity of type I receptors has been shown for other type I receptors; ALK-3 and ALK-6, upon co-expression with the *C. elegans* type II receptor Daf-4, were identified as type I receptors for both BMP-4 and OP-1. Furthermore, ActR-IA showed cross-reactivity with OP-1 in the presence of Daf-

4 (ten Dijke et al., 1994c). Recently, it has been shown that the *Xenopus* homologue of ALK-3 binds both BMP-2 and BMP-4 and that this receptor is involved in the induction of ventral mesoderm in the frog embryo (Graff et al., 1994).

The interpretation of the experiments in which type I and type II receptors were co-injected was made carefully. We considered the possibility that the synergistic effects could have been brought about by the simultaneous induction of two different pathways, which eventually could result in an enhanced effect. However, this does not explain the effects observed upon co-injection of ActR-IIB2 with ActR-IA or -IB, because ActR-IIB2 alone does not interfere with an endogenous signal transduction pathway. Furthermore, one can also imagine that the expression levels of the receptors in the cell membrane are so high that ligand independent complexes are formed. However, we show that the combined injection of TGFβR-I and ActR-IIB2 is not synergistic, indicating that the complexes formed are most likely ligand dependent. We conclude from our data that upon co-injection of type I and type II receptors, these overexpressed receptors do not depend on the presence of endogenous receptors, but only require an endogenous ligand to form an actively signaling complex. Most importantly, the induction of secondary axes coincides with the ectopic expression of several mesodermal markers, i.e. *goosecoid*, *no tail* and *axial*.

To quantify the induction of expression of *goosecoid* and *no tail* we performed an RNase protection analysis. For *no tail* we did not observe a significant induction of expression upon injection with the different receptors, which was to be expected; in the in situ hybridizations a clear ectopic expression was observed which was on average never more than twice the amount of normal expression. Taking into account that the frequency of abnormal embryos was always less than 100% (see Table 2) at most a twofold induction is to be expected, which is at the limit of detection in this experiment. The relative expression level of *goosecoid* over *no tail* as expressed in the last column of Table 3 indicates that the expression of *goosecoid* is 3–5-fold increased upon injection of embryos with ActR-IB in combination with each of the activin type II receptors, compared to the uninjected embryos, or embryos injected with other combinations of receptors. These results suggest that specific signaling pathways are activated by different combinations of receptors, resulting in subtle differences in the induction of mesodermal marker genes. Specificity of ActR-IB with respect to its down stream signaling will be studied in more detail in relation to an activin responsive element identified in the zebrafish *goosecoid* promoter (Joore et al., unpublished data). The exact signaling pathways activated by serine/threonine kinase receptors are so far unknown and will be the subject of future studies.

It is very tempting to speculate what the endogenous ligand(s) may be. The ligand(s) will belong to the TGFβ superfamily and activin, Vg1 and BMP-4 may be considered candidates. Recently, it has been demonstrated that truncated type II activin receptors abolish signaling of both Vg1 and activin in *Xenopus* animal caps (Schulte-Merker et al., 1994a). This lack of specificity of the activin receptors indicates that Vg1 as well as other members of the TGFβ superfamily are perhaps just as likely as activin to bind these receptors and thus to be involved in mesoderm induction in amphibians as has been postulated before (Thomsen and Melton, 1993; Dale et al., 1993) and more recently (Kessler and Melton, 1994, 1995). Moreover, Schulte-Merker et al., (1994b) have shown that overexpression of follistatin, an activin binding protein which blocks activin activity (Nakamura et al., 1990; Asashima et al., 1991) does not interfere with the signaling of ectopically expressed Vg1 in animal cap explants. Even though overexpression of follistatin induces neuralization of *Xenopus* animal caps, follistatin does not interfere with normal development of the *Xenopus* embryo, indicating that activin indeed may not be the endogenous mesoderm inducing factor (Schulte-Merker et al., 1994a; Kessler and Melton, 1994). Recently it has been shown that activin knock-out mice which lack both activin chains are not viable but develop normal mesoderm and form a complete, normal antero-posterior axis, excluding a major role for activin in these early processes (Matzuk et al., 1995). The data obtained with dominant negative variants of activin which interfere with mesoderm induction in medaka (Wittbrodt and Rosa, 1994) seem to contrast with the studies summarized above. However, it may be that the overexpressed dominant negative activin chains can form inactive dimeric ligands with other members of the TGFβ superfamily of growth factors and thus exert their effect on mesoderm induction.

Our study provides evidence that several type I and type II receptors which are members of the serine/threonine kinase receptor family are involved in early zebrafish development. However, the ligand(s) which interact in vivo with these receptors need to be identified. In the near future, zebrafish mutant analysis will probably shed new light on the role of the serine/ threonine kinase receptors and their specific ligands in early vertebrate development.

4. Experimental procedures

4.1. Plasmid constructs and in vitro transcription

All constructs used for SP6 RNA polymerase-dependent in vitro transcription contained the coding sequences in between the NcoI and SalI site of a modified pSP64T vector containing 5′ and 3′ untranslated regions of the *Xenopus* β-globin gene under control of the SP6 RNA polymerase promoter (Krieg and Melton, 1984).

The construct for mouse ActR-IIA contained a Kozak sequence 5′ of the start codon creating an NcoI site (GCCACCATGG) and an HA-epitope (Field et al., 1988) was inserted by PCR upstream from the stop codon followed by a SalI site. Truncated mouse ActR-IIA encodes the extracellular domain, the transmembrane domain, but only 8 intracellular amino acids, followed by the HA epitope, a stop codon and a SalI site. Full-length mouse ActR-IIB2 and ActR-IIB4 were carboxy terminally tagged with the KT3 epitope (MacArthur and Walter, 1984) and their truncated forms encode the complete extracellular and transmembrane domains and only four intracellular amino acids, followed by the KT3 tag. The mouse ActR-IA cDNA (Ebner et al., 1993) was extended with the HA epitope. The mouse ActR-IB cDNA (Verschueren et al., 1995) and the human TGFβR-I cDNA (Franzén et al., 1993) were inserted in the vector without untranslated regions. For RNA transcription, the constructs were linearized to include the 3′ *Xenopus* β-globin sequences. The RNAs were capped during in vitro synthesis.

4.2. Fish stocks and embryo injection

Zebrafish were maintained and raised as described (Westerfield, 1993) and embryos were obtained by natural matings. One-cell to 4-cell embryos, in their chorion, were mounted in agarose slots and injected with needles of ca. 5 μm width using a Narashige injection apparatus. A volume of ca. 1 nl, which was calibrated for each needle, with different concentrations of RNA (0–3600 ng/μl) was injected at the interphase between yolk and cytoplasm and embryos were grown at 28.5°C in 1/10 the Hank's solution (Westerfield, 1993). Embryos were either fixed for immunohistochemistry or in situ hybridization at 50–70% and 80–90% epiboly, respectively. Alternatively they were grown for 30 h, dechorionated and examined using a Zeiss Axiovert microscope equipped with differential interference contrast and recorded with a Sony optical memory disc recorder.

4.3. Whole mount in situ hybridization and immunohistochemistry

Whole mount in situ hybridizations were carried out as described previously by Joore et al. (1994). The *axial* probe used has been described by Strähle et al. (1993), the *goosecoid* antisense probe was made from a subcloned 252 bp RsaI fragment (bp 261–513) of the full-length cDNA cloned in pBSK–, linearized with EcoRI and synthesized with T3 RNA polymerase. For the *no tail* probe a PCR reaction was performed on the full-length cDNA (kindly provided by Dr Schulte-Merker) to obtain a fragment from bp 343 to 534 which was subcloned in pBSK–. After linearization of the plasmid with XbaI the RNA probe was synthesized with T7 RNA polymerase.

For immunostaining, embryos were fixed overnight at 4°C in 4% paraformaldehyde in PBS. After fixation embryos were rinsed with PBS, transferred to 100% methanol and stored at −20°C until use. Embryos were than gradually rehydrated from absolute methanol to PBS and rinsed with PBST (PBS, 0.1% Tween 20), incubated for 30 min at room temperature in acetone, transferred to 0.2 mM $CaCl_2$ for 5 min, again rinsed with PBST and incubated for 1 h in the blocking solution (PBST, 2% BSA). Incubation with the first antibodies was at 4°C overnight. Embryos were washed three times for 2 h each at room temperature in blocking solution and incubated overnight at 4°C with goat anti-mouse antibody coupled to FITC (Tago Inc.) or Cy3 (Jackson Laboratories). Embryos were washed in PBST, dehydrated through a graded series of methanol, cleared in Murray's (1/3 benzylalcohol and 2/3 benzylbenzoate) and analyzed by confocal laser scanning microscopy.

4.4. RNase protection assay

RNase protection analysis of *goosecoid* and *no tail* expression was carried out as described previously (Strähle et al., 1993). The ^{32}P probes were synthesized from the same constructs as applied in the in situ hybridization experiments. RNA was isolated from 10 injected embryos fixed at 80–90% epiboly in Trizol (GIBCO-BRL).

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References

Amaya, E., Stein, P.A., Musci, T.J. and Kirschner, M.W. (1993) Development 118, 477–487.

Asashima, M., Nakano, H., Shimada, K., Kinoshita, K., Ishii, K., Shibai, H. and Ueno, N. (1990) Roux's Arch. Dev. Biol. 198, 330–335.

Asashima, M., Nakano, H., Uchiyama, H., Sugino, H., Nakamura, T., Eto, Y., Ejima, D., Davids, M., Plessow, S., Cichocka, I. and Kinoshita, K. (1991) Roux's Arch. Dev. Biol. 200, 4–7

Attisano, L., Wrana, J.L., Cheifetz, S. and Massague, J. (1992) Cell 68, 97–108.

Attisano, L., Carcamo, J., Ventura, F., Weis, F.M.B., Massague, J. and Wrana, J.L. (1993) Cell 75, 671–680.

Bassing, C.H., Yingling, J.M., Howe, D.J., Wang, T., He, W.W., Gustafson, M.L., Shah, P., Donahoe, P.K. and Wang, X.-F. (1994) Science 263, 87–89.

Bhushan, A., Lin, H.Y., Lodish, H.F. and Kintner, C.R. (1994) Mol. Cell. Biol. 14, 4280–4285.

Carcamo, J., Weis, F.B., Ventura, F., Wieser, R., Wrana, J.L., Attisano, L. and Massague, J. (1994) Mol. Cell. Biol. 14, 3810–3821.

Dale, L., Howes, G., Price, B.M. and Smith, J.C. (1992) Development 115, 573–585.

Dale, L., Matthews, G and Colman, A. (1993) EMBO J. 12, 4471–4480.

Ebner, R., Chen, R.H., Lawler, S., Zioncheck, T. and Derynck, R. (1993) Science 262, 900–902.

Field, J., Ikawa, J.I., Broek, D., MacDonald, B., Rodgers, L., Wilson, L., Wilson, I.A., Lerner, R.A. and Wilger, M. (1988) Mol. Cell. Biol. 8, 2159–2165.

Franzén, P., ten Dijke, P., Ichijo, H., Yamashita, H., Schulz, P., Heldin, C.-H. and Miyazono, K. (1993) Cell 75, 681–692.

Graff, J.M., Thies, R.S., Song, J.J., Celeste, A.J. and Melton, D.A. (1994) Cell 79, 169–179.

Hammerschmidt, M. and Nusslein-Volhard, C. (1993) Development 119, 1107–1118.

He, W.W., Gustafson, M.L., Hirobe, S. and Donahoe, P.K. (1993) Dev. Dynam. 196, 133–142.

Helde, K.A. and Grunwald, D.J. (1993) Dev. Biol. 159, 418–426.

Hemmati-Brivanlou, A., Wright, D.A. and Melton, D. A. (1992) Dev. Dynam. 194, 1–11.

Hemmati-Brivanlou, A. and Melton, D.A. (1992) Nature 359, 609–614.

Jones, C.M., Lyons, K.M., Lapan, P.M., Wright, C.V. E. and Hogan, B.J.M. (1992) Development 115, 639–647.

Joore, J., van der Lans, G.B.L.J., Lanser, P., Vervaart, J.M.A., Zivkovic, D., Speksnijder, J.E. and Kruijer, W. (1994) Mech. Dev. 46, 137–150.

Kessler, D.S. and Melton, D.A. (1994) Science 266, 596–604.

Kessler, D.S. and Melton, D.A. (1995) Development 121, 2155–2164.

Koenig, B.B., Cook, J.S., Wolsing, D.H., Ting, J., Tiesman, J.P., Correa, P.E., Olson, C.A., Pecquet, A.L., Ventura, F., Grant, R.A., Chen, G.-X., Wrana, J.L., Massague, J. and Rosenbaum, J.S. (1994) Mol. Cell. Biol. 14, 5961–5974.

Kondo, M., Tashiro, K., Fuji, G., Asano, M., Miyoshi, R., Yamada, R., Muramatsu, M. and Shiokawa, K. (1991) Biochem. Biophys. Res. Commun. 181, 684–690.

Koster, M., Plessow, S., Clement, J.H., Lorenz, A., Tiedemann, H. and Knochel, W. (1991) Mech. Dev. 33, 191–200.

Krieg, P.A. and Melton, D.A. (1984) Nucleic Acids Res. 12, 7057–7070.

Lin, H.Y., Wang, X.-F., Ng-Eaton, E., Weinberg, R. A. and Lodish, H.F (1992) Cell 68, 775–785.

Letsou, A., Arora, K., Wrana, J.L., Simin, K., Twombly, V., Jamal, J., Staehling-Hampton, K., Hoffmann, F.M., Gelbart, W.M., Massague, J. and O'Connor, M.B. (1995) Cell 80, 899–908.

MacArthur, H. and Walter, G. (1984) J. Virol. 52, 483–491.

Mathews, L.S. and Vale, W.W. (1991) Cell 65, 973–982.

Mathews, L.S., Vale, W.W. and Kintner, C.R. (1992) Science 255, 1702–1705.

Mathews, L.S. (1994) Endocr. Rev. 15, 310–325.

Matzuk, M.M., Kumar, T.J., Vassalli, A., Bickenbach, J.R., Roop, D.R., Jaenisch, R. and Bradley, A. (1995) Nature 374, 354–356.

Nakamura, T., Takio, K., Eto, Y., Shibai, H., Titani, K. and Sugino, H. (1990) Science 247, 836–838.

Nishimatsu, S., Iwao, M., Nagai, T., Oda, S., Suzuki, A., Asashima, M., Murakami, K. and Ruberte, E., Marty, T., Nellen, D., Affolter, M. and Basler, K. (1995) Cell 80, 889–897.

Penton, A., Chen, Y., Staehling-Hampton, K., Wrana, J. L., Attisano, L., Szidonya, J., Cassil, J.A., Massague, J. and Hoffmann, F.M. (1994) Cell 78, 239–250.

Ruberte, E., Marty, T., Nellen, D., Affolter, M. and Basler, K. (1995) Cell 80, 889–897.

Schulte-Merker, S., Ho, R.K., Herrmann, B.G. and Nusslein-Volhard, C. (1992) Development 116, 1021–1032.

Schulte-Merker, S., Smith, J.C. and Dale, L. (1994a) EMBO J. 13, 3533–3541.

Schulte-Merker, S., van Eeden, F.J.M., Halpern, M.E., Kimmel, C.B. and Nusslein-Volhard, C. (1994b) Development 120, 1009–1015.

Smith, J.C., Price, B.M.J., van Nimmen, K. and Huylebroeck, D. (1990) Nature 345, 729–731.

Smith, J.C. (1993) EMBO J. 12, 4463–4470.

Stachel, S.E., Grunwald, D.J. and Myers, P.Z. (1993) Development 117, 1261–1274.

Strähle, U., Blader, P., Henrique, D. and Ingham, P. W. (1993).Genes Dev. 7, 1436–1446.

ten Dijke, P., Ichijo, H., Franzén, P., Schulz, P., Saras, J., Toyoshima, H., Heldin, C.-H. and Miyazono, K. (1993) Oncogene 8, 2879–2887.

ten Dijke, P., Franzén, P., Yamashita, H., Ichijo, H., Heldin, C.-H. and Miyazono, K. (1994a) Prog. Growth Factor Res. 5, 55–72.

ten Dijke, P., Yamashita, H., Ichijo, H., Franzén, P., Laiho, M., Miyazono, K. and Heldin, C.-H. (1994b) Science 264, 101–104.

ten Dijke, P., Yamashita, H., Sampath, T.K., Reddi, A.H., Estevez, M., Riddle, D.L., Ichijo, H., Heldin, C-.H. and Miyazono, K. (1994c) J. Biol. Chem. 269, 16985–16988.

Thomsen, G., Woolf, T., Whitman, M., Sokol, S., Vaughan, J., Vale, W. and Melton, D.A. (1990) Cell 63, 485–493.

Thomsen, G. and Melton, D. (1993) Cell 74, 433–441

van den Eijnden-van Raaij, A.J.M., van Zoelen, E.J.J., van Nimmen, K., Koster, C.H., Snoek, G.T., Durston, A. J. and Huylebroeck, D. (1990) Nature 345, 732–734.

Verschueren, K., DeWulf, N., Goumans, M.-J., Feijen, A., Grimsby, S., Vande Spiegle, K., ten Dijke, P., Morén, A., Vanscheeuwijck, P., Heldin, C.-H., Miyazono, K., Mummery, C., van den Eijnden-van Raaij, A.J.M. and Huylebroeck, D (1995) Mech. Dev. 52, 109–123.

Westerfield, M. (1993) The Zebrafish Book. University of Oregon Press, Eugene, OR.

Wilson, P.A. and Hemmati-Brivanlou, A. (1995) Nature 376, 331–333.

Wittbrodt, J. and Rosa, F.M. (1994). Genes Dev. 8, 1448–1462.

Wrana, J.L., Attisano, L., Wieser, R., Ventura, F. and Massague, J. (1994). Nature 370, 341–347.