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A carboxyl-terminal truncated version of the activin receptor mediates activin signals in early *Xenopus* embryos

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The function of a carboxyl-terminal truncated version of the *Xenopus* activin receptor, encoded by a previously isolated gene XSTK2, was investigated in early embryos. The transcript corresponding to the truncated receptor gene was detected throughout embryonic development although the temporal expression pattern was different from that of an intact receptor. Injection of XSTK2 mRNA into early embryos resulted in the formation of a duplicated body axis. Mesoderm induction as evaluated by the activation of the α-actin gene in presumptive ectoderm (animal cap) treated with exogenous activin was significantly enhanced by the injection of XSTK2 mRNA. These results suggest that the truncated receptor is capable of transmitting the activin signal to the same extent as the native receptor.

Activin; Ser/Thr kinase; Receptor; *Xenopus*; PCR

1. INTRODUCTION

Activin, a member of the TGF-β superfamily, is known to function not only as a regulator of hormone secretion [1,2] but also as a regulator of cell differentiation for a variety of cell types [3–5]. It has been demonstrated by affinity cross-linking that there are three molecular species of the activin receptor in murine erythroleukemia F5-5 cells and these have been designated as type I (42 kDa), type II (51 kDa) and type III (151 kDa) [6]. Recently, the structure of a mouse activin receptor has been investigated by gene cloning [7]. Based on the molecular weight predicted from the cDNA, the receptor was classified as a type II receptor. Furthermore, the type II receptor for TGF-β1 has also been cloned [8]. All the so far characterized type II receptors appear to possess protein Ser/Thr kinases in their cytoplasmic domains [9].

Similar activin receptor genes have previously been isolated from amphibian sources and these have been shown to be involved in morphogenesis during early development [10,11]. We have previously reported that there are at least four genes that encode activin receptor-like proteins in *Xenopus laevis* [12]. One gene was found to encode a receptor protein which lacks the carboxyl-terminal part of the Ser/Thr kinase located distal to domain VIII. We reasoned that the truncated receptor might represent a protein which plays a negative regulatory role in activin signaling. The present study was carried out in order to examine whether or not the truncated activin receptor was capable of correctly transmitting the activin signal. Here we report that the truncated receptor is functional and able to cause duplication of the partial body axis as well as enhancement of mesoderm induction to the same extent as the intact receptor.

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2. MATERIALS AND METHODS

2.1. *Reverse transcription-PCR (RT-PCR)*

In order to distinguish transcripts of the XSTK2 gene from those of the XSTK3 and XSTK8 genes, whose nucleotide sequences are highly similar to XSTK2 in the N-terminal region [12], RT-PCR was performed as described [13,14]. The sequences of the various oligonucleotide primers used are as follows. The upper strand primer sequence, 5′-GAAACAATGGCTCGTGGGC-3′, is located in a region in common to the XSTK2, XSTK3 and XSTK8 genes whereas the lower strand primer sequences, 5′-TCGCTGCACAAGTGATTACC-3′ and 5′-CTCTAGAACCTCAGGAGCC-3′, were chosen from regions specific to the XSTK2 and XSTK3/8 genes, respectively. The conditions used for PCR were as follows. Denaturation was performed at 94°C for 30 s followed by annealing at 60°C for 30 s with the subsequent extension reaction carried out at 72°C for 60 s. A total number of 30 reaction cycles were performed in the presence of [α-^{32}P]dCTP (Amersham). Template cDNA was synthesized with the use of MMLV reverse transcriptase (BRL) from total RNA purified from staged embryos by the AGPC method [15]. The PCR products were separated on 0.2 mm-thick polyacrylamide gels, which were then dried for autoradiography. For quantitation of muscle specific α-actin mRNA, we reverse transcribed total RNA isolated from animal cap explants and performed a PCR as previously described by Rupp et al. [16].

2.2. *In vitro transcription and translation*

Capped synthetic RNAs were generated as described [17]. The inserts of the *Xenopus* activin receptor clones XSTK2 (*Sac*II/*Xho*I insert of 0.7 Kb) and XSTK8 (*Bam*HI/*Xho*I insert of 1.5Kb) were subcloned

into a pSP73polyA vector which was constructed by the insertion of a 83 bp *Eco*RI/*Hin*dIII fragment containing a polyA tract and multiple cloning site region from pSP64polyA (Promega) into pSP73. The pSP73polyA based constructs were transcribed with T7 RNA polymerase in vitro. Each synthetic RNA was translated in a rabbit reticulocyte lysate (Promega) in the presence of [^{35}S]methionine (Amersham) and analyzed by SDS-PAGE.

2.3. *Injection of mRNAs*

Microinjection of receptor mRNAs, performed in order to study phenotypic alterations, was carried out essentially as described by Yuge et al. [18]. Briefly, mRNAs were injected into ventral blastomers of 4-cell embryos in Steinberg's solution after removal of the vitelline membrane. The embryos were then allowed to develop in the same solution for several days during the period of the experiment. For the mesoderm induction assay, injections were carried out according to the method of Moon et al. [19] in 5% Ficoll.

2.4. *Animal cap assay*

The animal cap assay using embryos injected with activin receptor mRNA was carried out as previously described [20].

3. RESULTS AND DISCUSSION

As previously reported, extensive screening of a *Xenopus* embryo cDNA library with mouse activin receptor cDNA [7] identified several activin receptor clones [12]. Interestingly, one of the clones designated as XSTK2 was found to encode a highly similar protein to that of intact receptors encoded by XSTK3 and XSTK8 but lacking the carboxyl-terminal part of the Ser/Thr kinase region located on the C-terminal side of domain VIII (Fig. 1A). The nucleotide sequence of XSTK2 contains a polyadenylation signal, AATAAA, followed by a polyA tract in the 3'-untranslated region consistent with the observation that the XSTK2 gene is transcribed. In our previous study, we have shown by Northern blot analysis that the XSTK2 and XSTK3 genes are transcribed in early embryos. However, we were unable to distinguish transcripts of XSTK2 from those of XSTK3 and XSTK8 since the probe was chosen from a region in common to the three receptor genes. In order to distinguish transcripts of XSTK2 from those of closely related intact receptor genes, oligonucleotide primer sets, each of which was specific to XSTK2 or XSTK 3/8, were designed (Fig. 1B). Using the respective primer sets, the transcript levels during early development were examined by RT-PCR. Fig. 2 shows an autoradiogram of the PCR products from XSTK2 and XSTK3/8. These products were of the expected size, *viz.* an XSTK2 product of 219 b.p. and the XSTK3/8 products of 242 b.p. It is evident that all genes are transcribed during early development. The transcript of XSTK2 appeared to increase as development proceed, whereas that of the intact receptor (XSTK3/8) appeared to fluctuate during early embryogenesis (Fig. 2) thus suggesting that the gene for the truncated receptor is independently regulated from that of the intact receptor gene. The temporal expression pattern of XSTK3/8 was consistent with our previous results obtained by Northern blotting [12].

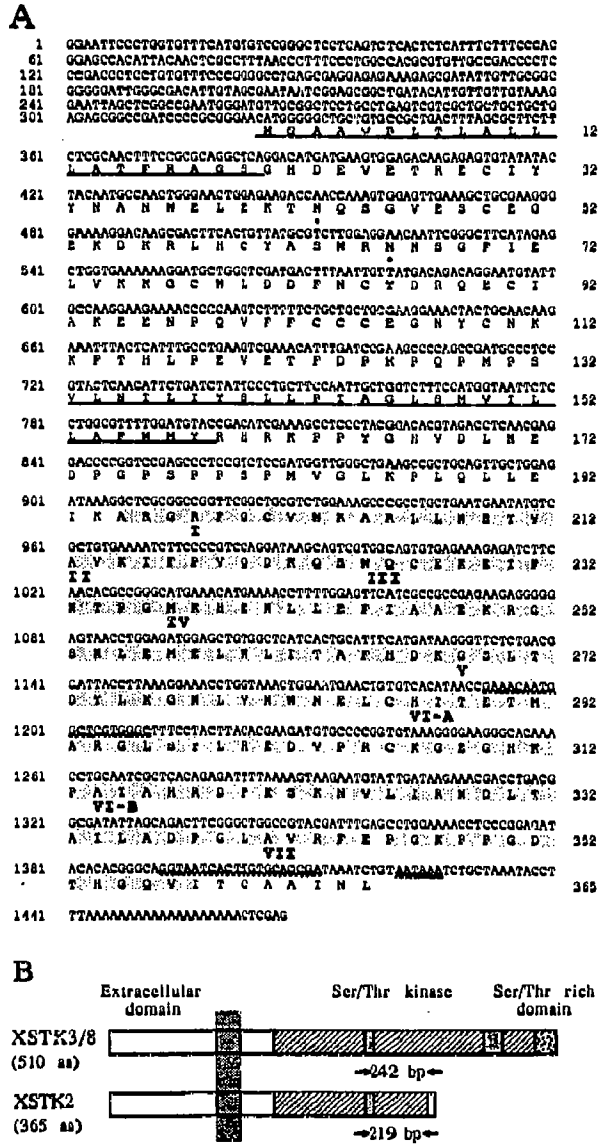

Fig. 1. Structure of the truncated activin receptor cDNA, XSTK2, and the deduced precursor protein. (A) The nucleotide sequence of the full-length XSTK2 cDNA clone is shown along with the deduced amino acid sequence of the putative receptor protein. The signal sequence and the transmembrane domain are highlighted by thin underlining. The sequences used to synthesize oligonucleotide primers for RT-PCR are indicated by wavy underlining. The putative polyadenylation site at the 3' end of the mRNA is denoted by double underlining. The Ser/Thr kinase domain is shown by shading of the amino acid sequence. Roman numerals under the deduced amino acid sequence refer to subdomains conserved among the various members of the protein kinase family. (B) Schematic representation of *Xenopus* activin receptor proteins. The extracellular domain, transmembrane region (shaded), Ser/Thr kinase domain and Ser/Thr rich domains are shown. Two characteristic inserts in the kinase domain are shaded [9]. The locations of the PCR primers are indicated by arrows and the predicted sizes of amplified products are also shown.

It was reasoned that the truncated receptor is capable of binding activin since a mouse activin receptor with an even larger carboxyl-terminal deletion has been observed to bind activin in a similar fashion to the intact

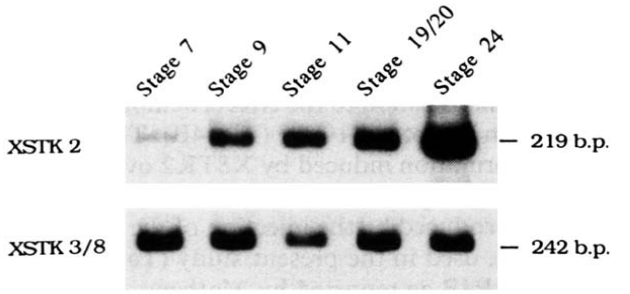

Fig. 2. RT-PCR analysis of the truncated and intact activin receptor mRNA during early development. 500 ng of total RNA from staged embryos was subjected to RT-PCR using specific 3′ reverse primers for truncated type (XSTK2) and intact type (XSTK3/8) receptor cDNAs. The upper strand primer lies in a region in common among the three receptor types. The transcript of EF-1α was amplified in a similar fashion in order to analyze both the quality and quantity of RNA (data not shown).

receptor [7]. Therefore, we speculated that the truncated receptor encoded by XSTK2 could bind activin but was not able to transmit activin signals because of the impaired kinase domain. In order to examine this hypothesis we looked at the effect of overexpression of both the truncated and intact receptor proteins. We employed

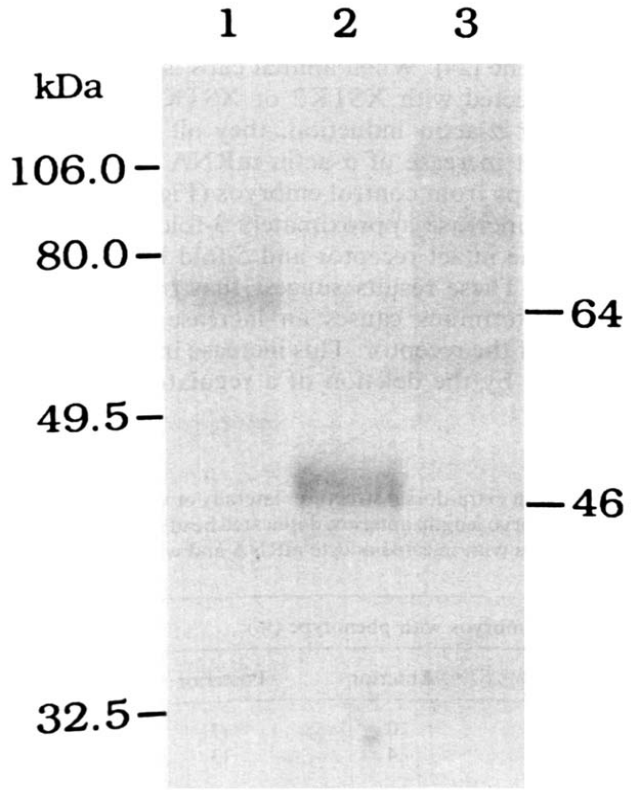

Fig. 3. In vitro synthesis of *Xenopus* activin receptor protein. The products produced by a nuclease-treated rabbit reticulocyte lysate were loaded onto a 10% SDS-PAGE. Lysate containing XSTK8 mRNA (lane 1), XSTK2 mRNA (lane 2) and lysate with no added RNA (lane 3). Molecular weight standards are indicated in kDa on the left.

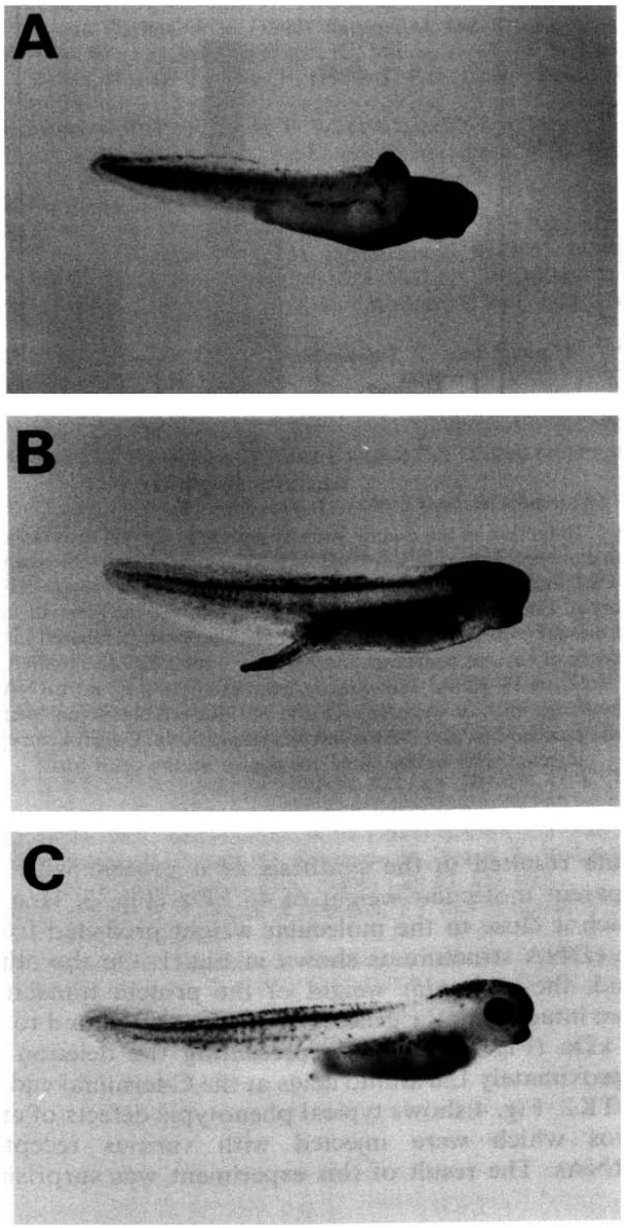

Fig. 4. Developmental defects in embryos injected with activin receptor XSTK2 and XSTK8 mRNAs. Embryos were injected at the 4-cell stage with mRNA synthesized in vitro (900 pg mRNA/embryo). (A) represents an embryo injected with XSTK8 mRNA and (B) represents an embryo injected with XSTK2 mRNA. A control embryo injected with buffer alone is shown in (C).

the strategy of injecting XSTK2 mRNA into early embryos since it has been previously demonstrated that injection of intact activin receptor mRNA results in duplication of the body axis [10,11] and an increased level of responsiveness of animal cap explants to activin [11]. To ascertain the molecular weight of receptor proteins that would be translated by injected mRNAs, we first translated these mRNAs using a rabbit reticulocyte lysate (Fig. 3). The addition of XSTK2 mRNA to the

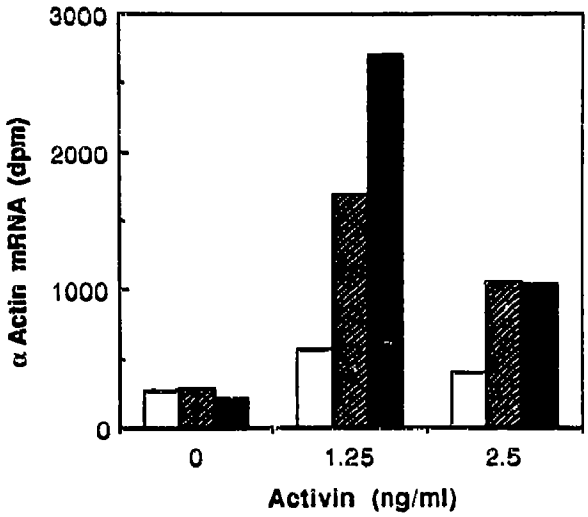

Fig. 5. Induction of the α-actin gene by activin in animal caps isolated from embryos. Embryos were injected at the 4-cell stage with receptor XSTK2 and XSTK8 mRNAs synthesized in vitro (400 pg RNA/embryo). Embryos were then allowed to develop to the blastula stage and animal caps obtained from five blastulae were incubated in the presence of various concentrations of activin for 1 day and pooled for the isolation of RNA. The relative expression of α-actin mRNA in animal caps injected with XSTK8 and XSTK2 mRNA is represented by the hatched box and the shaded box respectively. Control embryos injected with buffer alone are shown by the open box.

lysate resulted in the synthesis of a protein with an apparent molecular weight of 46 kDa (Fig. 3, lane 2) which is close to the molecular weight predicted from the cDNA structure as shown in Fig. 1. On the other hand, the molecular weight of the protein translated from intact receptor gene, XSTK8, was estimated to be 64 kDa (Fig. 3, lane 1) representing the deletion of approximately 120 amino acids at the C-terminal end of XSTK2. Fig. 4 shows typical phenotypic defects of embryos which were injected with various receptor mRNAs. The result of this experiment was surprising in that overexpression of the carboxyl-terminal truncated receptor lead to significant alterations in normal development with the production of a duplicated body axis in a similar fashion to the effects demonstrated with intact activin receptors [10,11] (Fig. 4B). The frequency of the malformation induced by XSTK2 overexpression was approximately 13% and this percentage is comparable to that produced by the injection of the intact receptor, XSTK8, used in the present study (Table I) and to that of xActRIB as reported by Mathews et al. [11]. In contrast to the effect observed with *Xwnt*-8, a gene which induces complete body axis formation [21,22], the additional body axis formed by injection of XSTK2 mRNA is incomplete (Fig. 4A en B) and occurs partially along the antero-posterior axis. It is interesting to note that such a bifurcation produced by the truncated receptor occurs exclusively in the posterior region which includes the tail while overexpression of the intact receptor often results in duplication of the anterior region.

Next, we examined the effect of overexpression of the truncated receptor on the competence of animal cap cells to respond to exogenous activin. It is well known that the animal cap of the blastula can respond to exogenous activin. It is well known that the animal cap of the blastula can respond to activin and thereby differentiate into mesodermal derivatives [20,23]. This differentiation is associated with the activation of mesodermal marker genes such as *MyoD* [16] and the muscle specific α-actin gene [24]. When animal caps isolated from embryos injected with XSTK2 or XSTK8 mRNAs were tested for α-actin induction, they all demonstrated a significant increase of α-actin mRNA as compared to animal caps from control embryos (Fig. 5). This induction was increase approximately 3-fold by overexpression of the intact receptor and 5-fold by the truncated receptor. These results suggest that truncation of the carboxyl-terminus causes an increase in the intrinsic activity of the receptor. This increase in activity may be explained by the deletion of a regulatory region, such

Table I

Defects produced in embryos injected with *Xenopus* activin receptor RNAs. Embryos with extra-dorsal structures laterally or ventrally placed were classified into several groups based on appearance, viz. normal, no defect, short, short embryo length; anterior, duplicated head structures; posterior, extra tail- and trunk-like protrusions; spina bifida, bifurcation at posterior end. Controls with injected oocyte mRNA and with no injected RNA are presented for comparison.

	RNA injected (pg/embryo)	Total (*n*)	Embryos with phenotype (%)				
			Normal	Short	Anterior	Posterior	Spina bifida
XSTK8	500	66	68	5	0	17	11
	900	47	72	0	4	13	6
XSTK2	500	63	78	2	0	14	6
	900	42	90	0	0	7	2
Oocyte mRNA	900	12	92	0	0	0	8
Mock		26	88	0	0	0	11

as an autophosphorylation site(s), which may attenuate receptor kinase activity. This is consistent with observations relating to the c- *erb*B-2 gene in that the lack of an autohosphorylation site located close to the carboxyl-terminus enhances the kinase and transforming activities of ErbB-2 [25].

In summary, the present study of the effects of receptor overexpression on morphogenesis and α-actin gene activation suggests that the truncated activin receptor can mediate activin signals and function in a similar manner to the intact receptor. Precise investigations focusing on structural-functional relationships of the activin receptor are necessary in order to determine the active core of the Ser/Thr kinase and to correlate atophosphorylation of the receptor with various biological phenomena.

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