Autoinduction of activin genes in early *Xenopus* embryos

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Activin exhibits a potent mesoderm inducing activity towards the ectodermal tissue (animal cap) of *Xenopus laevis* blastulae. Thus in order to investigate the role of activin in morphogenesis of early *Xenopus* embryos, activation of genes for activin β A and β B was examined by the reverse transcription polymerase chain reaction. *In vivo*, activin β B mRNA appears to be present in embryonic stage 1 whereas β A mRNA is undetectable prior to gastrulation. β B and β A mRNAs were noted to accumulate after stages 9 and 15 respectively. Activin gene expression in *Xenopus* animal caps was examined after treatment with various concentrations of activin A. Under these treatment conditions, both

activin β A and β B mRNAs accumulated in a dose-dependent fashion after 24 h. The same effect was noted for treatment with similar concentrations of activin B. Accumulation of mRNAs was inhibited by the addition of cycloheximide to the culture medium, consistent with the proposition that activin gene expression requires certain protein factors. In total, therefore, these data suggest that an autoinduction mechanism is involved in the regulation of activin mRNA levels in normal *Xenopus* embryos and that this mechanism may play a pivotal role during early embryonic development.

INTRODUCTION

Activin, a 25 kDa dimeric protein, was originally discovered by its ability to stimulate the secretion of follicle-stimulating hormone from cultured anterior pituitary cells [1,2]. Later, activin was found to be identical to erythroid differentiation factor, which has the capability of stimulating erythroleukaemia cells lines such as F5-5 and K562 to differentiate into haemoglobin-synthesizing cells [3,4]. Because the primary structure of the activin protein is highly conserved among various species and is also highly related to the structure of transforming growth factor (TGF)- β , activin has been included in the TGF- β superfamily [5]. Many other biological activities demonstrated in a variety of cell types and tissues have subsequently been reported for this protein [6–10]. Therefore, activin has been considered as being a multi-functional growth factor which is able to regulate cellular differentiation in vertebrates [11]. So far, three subtypes of activin have been characterized: activin A [1], activin B [12] and activin AB [2]. All three activins are dimeric proteins, held together by disulphide bridging of two translation products derived from the activin β A and β B genes [13].

A number of workers have shown that activin A has potent mesoderm inducing activity in the African crow frog, *Xenopus laevis* [14–17]. This protein induces the muscle-specific α -actin gene and other mesodermal marker genes such as MyoD to promote mesodermal formation [18]. Additionally, activin A by itself is able to induce a variety of mesodermally derived cell types and tissues, including various blood cells, muscle tissue and notochordal tissue from animal caps of *Xenopus* blastulae in a concentration-dependent fashion [19,20]. Recently *Xenopus* activin genes encoding proteins highly identical in sequence to mammalian activins have been isolated [15,21]. Interestingly, over-production of the *Xenopus* activin molecule initiated through microinjection of its corresponding mRNAs resulted in duplication of the body axis [15]. Furthermore, a transcript of the *Xenopus* homeobox gene *gooseoid*, whose distribution is

closely correlated with Spemann's organiser, has been shown to accumulate rapidly in animal caps explants after treatment with activin [22].

Thus, evidence suggests that activin has a central role in the process of pattern formation during early *Xenopus* development [23]. This has prompted us to investigate the regulation of the activin genes in the period of early *Xenopus* embryonic development. First, in order to study gene regulation of activin, we have examined endogenous levels of mRNAs for activin β A and β B using the reverse transcription (RT)-PCR. Subsequently, we have further characterized the effect of the activin protein itself in the regulation of activin gene expression.

In this present study, we demonstrate that activin genes can be induced by activin proteins in an autocrine fashion. The physiological significance of this auto induction mechanism present during early embryogenesis is discussed concomitant with our findings.

MATERIALS AND METHODS

Materials

Activin A was produced from recombinant human activin A expressed in Chinese hamster ovary cells [4] and purified to homogeneity. Activin B was isolated from porcine follicular fluid [24]. Cycloheximide (CHX) and all-*trans*-retinoic acid (RA) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). CHX was prepared as a 10 mg/ml stock solution in ethanol. RA was prepared as a 10^{-2} M stock solution in dimethyl sulphoxide. Reagents listed above were diluted to the required concentration in $1 \times$ Steinberg's solution [14] before use.

Embryo manipulations

Preparation of animal caps from *Xenopus* blastulae was performed as previously described [14]. Briefly, *X. laevis* embryos were obtained by artificial fertilization [25]. The jelly coat was

Abbreviations used: TGF, transforming growth factor; RT, reverse transcription; CHX, cycloheximide; RA, retinoic acid; MMLV, Moloney murine leukaemia virus; EF, elongation factor.

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removed with 2% (w/v) cysteine-HCl, pH 7.8, with the various embryonic stages classified according to previous literature [26]. The vitellin membrane was removed manually using forceps and embryos were dissected with a tungsten needle in agar-coated plastic dishes in Steinberg's solution. Animal cap cells were removed during embryonic developmental stage 9 and cultured in Steinberg's solution with or without the inclusion of purified activin proteins.

RNA isolation and RT-PCR assay

Total RNA was prepared using the acid guanidinium-thiocyanate phenol/chloroform extraction method as previously described [27] with the following modifications. Ethanol-precipitated RNA was dissolved in proteinase K buffer [50 mM Tris/HCl, pH 7.4; 50 mM NaCl; 5 mM EDTA and 0.5% (w/v) SDS] containing 0.2 mg/ml of proteinase K (Merck) and incubated at 37 °C for 1 h. After phenol/chloroform extraction, RNA was precipitated with ethanol and dissolved in diethyl pyrocarbonate-treated water.

The RT-PCR assay was performed according to a standard procedure [28]. Total RNA was transcribed into cDNA by Moloney murine leukaemia virus (MMLV) RT in the following fashion. A 20 µl volume reaction mixture consisting of 100–500 ng total RNA, RT buffer (50 mM Tris/HCl, pH 8.3, 75 mM KCl, 3 mM MgCl₂), 100 pmol of random hexamer primers, 0.5 mM each dNTP, 10 mM dithiothreitol, 20 units of RNasin (Promega) and 100 units of MMLV RT (BRL) was incubated at 37 °C for 60 min followed by heating to 95 °C for 10 min. PCR was performed in a total reaction volume of 10 µl which included PCR buffer (10 mM Tris/HCl, pH 8.8, 50 mM KCl, 1.5 mM MgCl₂, 0.1% (v/v) Triton X-100) in addition to 0.2 mM each dNTP, 5 µCi of [α -³²P]dCTP (3000 Ci/mmol), 5 pmol of each primer and 0.2 units of Taq DNA polymerase (Wako Chemicals, Japan). The mixture was overlaid with 15 µl of mineral oil and incubated in an auto-thermal cycler (Perkin-Elmer-Cetus); denaturation was performed at 95 °C for 30 s, primer annealing at 62 °C for 30 s and the subsequent extension reaction at 72 °C for 1 min. A 4 µl aliquot of the amplified PCR sample was electrophoresed on a 5% (w/v) polyacrylamide gel and the resultant PCR fragment visualized by autoradiography. To quantify the relative amounts of amplification products, chosen bands were excised from dried gels and the radioactivity determined by Čerenkov counting. EF1- α was used as a positive control to demonstrate the fidelity of the RT and to assess the intactness of the isolated RNA [29].

The following sets of primers complementary to *Xenopus* activin cDNA clones were synthesized on a Cyclone Plus DNA synthesizer (Milligen/Bioscience) and subsequently used for the PCR:

Activin β A (5') 5'-ACCACCAGCAAAGAGGTCAGAAAG-3'

Activin β A (3') 5'-GTCTTCATCGTCCTTCTTCTTC-3'

Activin β B (5') 5'-ACAGGAAGTTCATTGTGATCCCTC-3'

Activin β B (3') 5'-TGGTTGGTCACTTGAGAATGAAGG-3'

EF-1 α (5') 5'-CCTGAATCACCCAGGCCAGATTGGTG-3'

EF-1 α (3') 5'-GAGGGTAGTCTGAGAAGCTCTCCACG-3'

RESULTS

Detection of activin transcripts by RT-PCR

Pairs of oligonucleotide primers designed for both *Xenopus* activin β A (A. Suzuki, S.-I. Nishimatsu, K. Murakami and N. Ueno, unpublished work) and β B cDNAs [30] were used for

RT-PCR. In order to prevent cross-binding, each set of primers was selected from regions that shared minimal similarity between the activin genes (Figure 1). PCR products amplified by each set of primers using embryonic stage 24 cDNA as a template were subcloned into pCR1000 (Invitrogen) and nucleotide sequences were determined by the dideoxy chain termination method [31] in order to confirm that the amplified products were of the expected sequence in each case (results not shown). The results obtained demonstrated that the sequences of the PCR amplicants matched identically with those of the *Xenopus* activin β A and β B genes. In order to perform quantitative PCR analyses, it is prerequisite to determine optimal PCR cycles in which PCR products are increased exponentially [28]. Therefore, PCR cycle-number dependency of amplified products was examined using stage 24 cDNA by increasing reaction cycles (20–40 cycles). β A and β B transcripts were amplified in an exponential manner during cycles 26–30 and 34–38, respectively. Thus, we have determined the optimal number of reaction cycles for β A and β B to be 28 and 36 respectively, using stage 24 cDNA (results not shown). The use of a different number of cycles for each mRNA is necessitated by the difference in the abundance of the two mRNAs in stage 24 embryos (results not shown).

To ascertain the primer specificity, we examined the detection of transcripts for activin β A and β B during early embryogenesis under the quantitative PCR conditions as described above. As shown in Figure 2(a), activin β A mRNA was first detected in the stage 15 embryo (early neurula development) and these levels were maintained during the period of later development. Activin β B mRNA (Figure 2b) was detected at much earlier stages of development than β A mRNA and reached significant levels at developmental stage 9, although comparison of relative amounts of β A and β B mRNAs cannot be made because different numbers of PCR cycles were used for β A and β B transcript amplification.

Nevertheless, these results are consistent with previous observations [15] except for the presence of β B mRNA before stage 9. Thus, the RT-PCR has proven to be a useful method for detecting β A and β B mRNAs independently and for monitoring the level of each transcript during development. The possibility that genomic DNA was amplified in this experiment can be excluded because no PCR product was detected in the absence of RT (results not shown).

Autoinduction of activin genes in isolated animal caps

In order to investigate the mechanism for developmental regulation of activin genes by growth factors, we first examined the effect of activin protein addition. As stated above, activin β B

Figure 1 Primers used for RT-PCR

The structures of the precursor polypeptides are represented by horizontal bars. The prepro and mature regions are represented by open and closed bars respectively; the 5'- and 3'-noncoding sequences are indicated by lines. Each primer set, denoted by arrows, was designed specifically to detect β A and β B transcripts. The primer sets for β A and β B transcripts produce amplified products comprising 248 bp (positions 558–805) and 301 bp (positions 1240–1540) respectively.

Figure 2 Levels of activin transcripts during early *Xenopus* embryogenesis

Total RNA (500 ng) from staged *Xenopus* embryos was subjected to RT-PCR using specific primers for activin β A and β B. Cycle numbers of 28 and 36 were used for activin β A (a) and β B (b), respectively. The Nieuwkoop developmental stage is indicated at the top of each plate.

mRNA appears in the animal cap from stage 9 of embryonic development. Therefore, we examined the effect of incremental dosages of activin proteins incubated with cultures of animal caps from this developmental stage. After 36 h, animal caps were harvested for RNA extraction and subsequent analysis. Addition of the activin A protein resulted in the accumulation of activin mRNAs in a dose-dependent fashion, as illustrated in Figure 3. The EC_{50} concentration, estimated from four independent experiments, was ~ 2 ng/ml for induction of both activin mRNAs. The recorded maximal rate of activin gene induction at 5 ng/ml of added protein most likely reflects a physiological plateau rather than an artifact of the PCR reaction itself, as repetition of the experiment using a greater number of PCR cycles resulted in more incorporation of radioactivity in the PCR products (results not shown).

It is not clear whether the small drop in both β A and β B mRNA levels caused by the addition of 10 ng/ml of activin A (Figure 3) has any physiological significance or not. This observation may be explained by invoking a threshold phenomenon for the activin protein concentration necessary to produce an autoinduction effect, as has been previously suggested for α -actin expression [19]. Alternatively, the phenomenon may be related to RNA stability or the efficiency of RNA purification, as the level of elongation factor (EF)-1 α is not even. The same experiment was repeated three times using various unrelated batches of embryos, with similar results being obtained in all cases.

Various time-course effects of activin A on β A and β B mRNA induction were examined in animal caps cultured in the presence of 5 ng/ml activin protein. Total RNAs were extracted from each batch in order to estimate activin mRNA levels. A concentration of 5 ng/ml activin A was chosen because we have already demonstrated that the muscle-specific actin gene expression is maximally induced by treatment with 2.5–5.0 ng/ml activin A, with the autoinduction of activin mRNA reaching a maximum at 5.0 ng/ml (Figure 3).

As shown in Figure 4, both activin β A and β B mRNAs were markedly induced after 24 h of activin A treatment. An approximately 5-fold increase in the level of activin mRNAs compared

Figure 3 Dose-dependent stimulation of activin gene expression induced by activin A

(a) Animal caps were treated with different concentrations of activin A for 36 h. Total RNAs were extracted from animal caps and subjected to the RT-PCR assay. To assure the quality and normalize the quantity of the various RNA extracts, an RT-PCR assay for EF-1 α was additionally performed. Concentrations of activin A used in this analysis are shown in the top row. (b) The relative amounts of activin transcripts were quantified by measuring the radioactivity of the corresponding bands (see the Materials and methods section). The ratios of the radioactivity from each induction point to that recorded at maximum induction are plotted against respective concentrations of activin A. (○) β A mRNA; (●) β B mRNA.

Figure 4 Time-course effects of activin A on activin and α -actin gene expression

Animal caps were excised from blastula embryos at stage 9 and treated with or without recombinant human activin A (5 ng/ml) for the indicated time periods (0–36 h), before being subjected to RNA extraction and subsequent RT-PCR assay.

Figure 5 Response of activin gene expression induced by activin A

(a) Animal caps were treated with 5 ng/ml activin A for chosen time periods ranging from 0 to 120 min, washed twice with Steinberg's solution and then cultured for an additional 36 h in unsupplemented medium. mRNA was extracted and used for RT-PCR. (b) The relative amounts of activin transcripts were quantified by measuring the radioactivity of the corresponding bands (see the Materials and methods section). The ratios of the radioactivity from each induction point to that recorded at maximum induction are plotted against period of incubation with activin A. $\circ$, βA mRNA; $\bullet$, βB mRNA.

with basal expression was observed on the basis of the proportional radioactive incorporation into the respective PCR products. Similarly, the α -actin gene was induced by treatment with activin; however, time-course effects of α -actin gene induction were found to be different from those of activin mRNAs because α -actin gene expression was detected after 12 h of activin treatment (Figure 4) whereas βA and βB mRNAs were undetectable under similar conditions. These data are consistent with a more immediate response in the expression of the α -actin gene to activin addition.

Figure 5 demonstrates activin gene induction upon the exposure of animal caps to activin for different time intervals followed by their culturing in the absence of activin for an additional 36 h. The results show that a 30–60 min exposure of animal caps to activin is sufficient to stimulate accumulation of both activin mRNAs, which implies that activin can interact with its receptor within 60 min to trigger the signal-transduction pathway. This observation is in accord with previous results [20] which have shown that a 60 min exposure to 5 ng/ml of purified

Figure 6 Effects of CHX and RA on activin gene expression as induced by activin A

(a) Animal caps were induced by activin A with or without CHX as follows. Animal caps were preincubated in Steinberg's solution containing 5 μ g/ml CHX and then treated with 5 ng/ml activin A and 5 μ g/ml CHX for 90 min. After washing with Steinberg's solution, animal caps were cultured for a further 36 h and then subjected to mRNA extraction and RT-PCR. (b) Animal caps were treated with 5 ng/ml activin A and 10^{-6} M RA for 90 min. After washing with Steinberg's solution, the animal caps were incubated for an additional 36 h and then subjected to mRNA extraction followed by RT-PCR.

XTC-mesoderm-inducing factor, a highly purified activin preparation from XTC cells, was sufficient to induce animal cap mesodermal differentiation.

The prerequisite for protein synthesis in the autoinduction of activin genes was demonstrated by the use of CHX. In this experiment, stage 9 animal caps were preincubated for 30 min with 5 μ g/ml CHX followed by a 90 min incubation with 5 ng/ml activin A in addition to 5 μ g/ml CHX. Tissues were subsequently rinsed and cultured in non-supplemented medium for an additional 36 h. This treatment is known to reduce protein synthesis by 75–95% [22,32]. As shown in Figure 6(a), induction of activin genes was completely abolished by treatment with CHX. Similarly, activation of the α -actin gene was also completely inhibited by CHX (results not shown). As an internal control, CHX treatment did not affect the levels of EF-1 α mRNA, indicating that inhibition of activin and α -actin gene activation is not the result of any intrinsic animal cap cellular damage.

The above results suggest that activin gene expression requires *de novo* protein synthesis. However, in sharp contrast to the activin gene, induction of the *Xenopus* homeobox genes *gooseoid* [22], *Mix.1* [32], *Xlim-1* [33] and *XFKH1* [34] is relatively rapid. These genes are strongly expressed in animal caps within a few hours of exposure to activin. In addition, experiments with CHX have revealed that the induction of these genes does not require prior protein synthesis [22,32–34].

In this context, it was important to examine whether or not autoinduction of activin genes could be, at least partially, mediated by the prior translation of activin-responsive genes which may encode transcriptional factors such as those produced by the homeobox genes. RA is known to mediate transcriptional regulation, including the homeobox genes which are essential for pattern formation in animal development [35]. In *Xenopus*, treatment of early embryos with RA results in head defect formation [36–38]. In addition, RA acts on mesoderm and disturbs pattern formation of the anterior–posterior axis [39], in contrast to activin which plays an important role in generating normal axis development [23]. Hence, in order to examine whether

activin autoinduction is affected by RA treatment, animal caps were cultured in the presence of 10^{-6} M RA; a concentration which has been shown to perturb head formation [36]. Figure 6(b) shows the effect of RA on autoinduction of the activin genes. Co-incubation of activin A with RA completely inhibited autoinduction of the activin β A and β B genes, whereas EF-1 α gene expression, used as a control, was unaffected. These results also suggest that autoinduction may be mediated by biosynthesis of *goosecoid*, as rapid induction of this gene in *Xenopus* animal caps has been reported to be inhibited by concentrations of RA similar to those used in our present studies [22].

All of the experiments described in this paper were repeated using activin B protein purified from porcine follicular fluid, with similar results being observed in each case.

DISCUSSION

In the present work, we have successfully employed RT-PCR [28,40] to both demonstrate the presence of and quantify levels of activin mRNAs in *Xenopus* embryos. These data assist in resolving the controversial question of whether activin is able to function as an endogenous mesoderm-inducing factor in *X. laevis*. Although it has been shown that activin-like activity can be detected in unfertilized eggs [41] and blastulae at trace levels, it is still possible that activin may not be directly involved in mesodermal induction in embryos as no activin mRNA has ever been detected in embryos during the induction phase. Our present analyses have indicated that the activin β B gene is transcribed and probably translated into protein in early embryos, suggesting that the activin B protein may play a role in induction. Detection of activin mRNA is made possible by the use of RT-PCR in order to detect mRNAs as compared to conventional Northern blot or ribonuclease protection analyses which have been utilized previously in order to detect activin mRNAs in *Xenopus* embryos [15].

In our approach to investigating the regulation of the activin genes, activin proteins were chosen to be incubated with animal caps from *Xenopus* blastulae because it has been reported previously that the gene for TGF- β 1, whose structure is related to activin, is positively regulated by TGF- β 1 protein [42], suggesting that a similar situation may well apply to activin gene regulation. Moreover, two additional growth factors, platelet-derived growth factor [43] and TGF- α [44], have also been reported to be induced in an autocrine fashion. As expected, activin genes are induced by activin in a dose-dependent manner within animal caps. We have therefore speculated that activin protein signalling is essential for pattern formation in early development and is responsive to activin levels within the cell [41] via an autoinduction mechanism. Furthermore, our results are consistent with the notion that cellular receptors for both activin A and activin B are present on the surface of animal caps cells and are functional.

Recently, activin receptor genes encoding serine/threonine kinases have been cloned from *Xenopus* and their contribution in morphogenesis of early *Xenopus* embryos has been examined [45–48]. Like activin, activin receptors also appear to be involved in the formation of the anterior–posterior body axis, as transient overexpression of activin receptor mRNAs causes duplication of the body axis [45,46,48]. Although the specificity of the four activin receptor subtypes so far identified remains speculative, all of these subtypes are expressed throughout early embryonic development. Autoinduction of activin genes is putatively considered to be triggered through phosphorylation of certain critical cytoplasmic protein(s) by the direct action of activin receptors.

In order to explore further signal-transduction pathways, it is important to investigate the nuclear proteins responsible for autoinduction of the activin genes, including activin-inducible transcriptional factors [22,32–34]. Additionally, analysis of the promoter regions corresponding to the activin genes will be required to identify *cis*-acting determinants necessary for activin signalling.

One particularly interesting finding from our present work is that activin autoinduction is inhibited by RA. This is presumed to block normal development of structural head formation [36] through binding to specific nuclear receptors which have been identified in *Xenopus* [49]. It has been demonstrated not only in *Xenopus* but also in the chick that implantation of an RA-absorbed bead into the anterior limb bud results in duplication of the limb digit [50]. Therefore, RA can be considered as a potential candidate for a key morphogenic molecule that regulates normal pattern formation in animal development. It has been extensively reported in the literature that the expression of homeobox genes is regulated by RA in a variety of cell types [35]. It is interesting that a number of homeobox genes such as *Xlim-1* are positively regulated whereas other homeobox genes are negatively regulated. *goosecoid* is one of the genes whose expression is decreased by treatment with RA. Assuming that homeobox genes such as *goosecoid* are involved in autoinduction mechanisms, RA may cause head formation defects by suppressing *goosecoid*, which in turn inhibits autoinduction of the activin genes. Further detailed analyses of the activin autoinduction mechanism in various developmental processes remain a high priority for future studies.

We thank Drs. Douglas A. Melton and Gerald Thomsen for providing nucleotide sequence information of the *Xenopus* activin β B subunit gene, Dr. Reiko Makino for RT-PCR technical advice, and Drs. Nicholas Ling and John A. Loudon for critical reading of this manuscript. This study was supported by grants-in-aid for Scientific Research from the Ministry of Education, Science and Culture of Japan and a grant from the Chichibu Cement Co.

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