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Truncated Activin Type II Receptor Inhibits Erythroid Differentiation in K562 Cells

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Abstract Two receptor serine/threonine kinases (types I and II) have been identified as signaling transducing activin receptors. We studied the possibility of inhibiting activin A-dependent differentiation in K562 cells, using a dominant negative mutant of type II receptor. A vector was constructed expressing activin type II truncated receptor (ActRIIa) that lacks the cytoplasmic kinase domain. Since activin type I and II receptors form heteromeric complexes for signaling, the mutant receptors compete for binding to endogenous receptors, hence acting in a dominant negative fashion. K562 cells were stably transfected with ActRIIa, and independent clones were expanded. The truncated cDNA was integrated into the genome of the transfectants, as shown by polymerase chain reaction; and the surface expression of truncated receptors was shown by affinity cross-linking with ^{125}I-activin A. In wild-type K562 cells, activin A induced erythroid differentiation and cells started to express hemoglobins. In transfected cells expressing ActRIIa, the induction of erythroid differentiation was abrogated and less than 10% of cells were hemoglobin-containing cells after culture with activin A. Further transfection with wild-type type II receptors rescued the mutant phenotype of these transfectants, indicating that the effect of ActRIIa is dominant negative. In addition, phosphorylation of the cytoplasmic kinase domain of the type II receptor in vitro confirms the autophosphorylation of this portion of the receptor. Therefore, induction of erythroid differentiation in vitro is mediated through the cell surface activin receptor, and interference with this receptor signaling inhibits this process of differentiation in K562 cells. J. Cell. Biochem. 78:24–33, 2000.

Key words: dominant negative regulation; type II receptor; activin A; K562

Activins act on multiple tissues and play a variety of roles in cellular function and proliferation [Vale et al., 1990]. Activins are evolutionarily conserved proteins that belong to the transforming growth factor-β (TGF-β) superfamily of growth and differentiation factors [Mason et al., 1985]. Activin A (β_A β_A), a homodimeric form of activin [Vale et al., 1986; Ling et al., 1986], is produced in large amount by bone marrow [Shao et al., 1992b; Yamashita et al., 1992] and exerts significant effects on proliferation and differentiation of erythroid progenitor cells [Yu et al., 1987, 1989; Broxmeyer et al., 1988].

Subsequent studies have identified a family of transmembrane serine/threonine kinases that act as receptors for activins and TGF-β [reviewed in refs. [Ying et al., 1997; Massague, 1998; Mathews, 1994]. These receptors are divided into two distinct subfamilies, type I and type II, which are distinguished by the level of sequence homology of their kinase domains and by other structural and functional features. The first receptor cloned was the cDNA of murine activin type II receptor, which encoded a transmembrane protein comprising a ligand-binding extracellular domain and an intracellular kinase domain with predicted serine/threonine specificity [Mathews and Vale, 1991]. It has also been shown that type I and II receptors for activin and TGF-β act cooperatively to bind ligands and transduce sig-

Abbreviations used: ActRII, full-length activin type II receptor; ActRIIa, truncated activin type II receptor lacking kinase domain; BSA, bovine serum albumin; DTT, dithiothreitol; FCS, fetal calf serum; GST, glutathione S-transferase; IPTG, isopropyl-β-D-thiogalactopyranoside; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; PVDF, polyvinylidene difluoride; SDS, sodium dodecyl sulfate; TGF-β, transforming growth factor β.

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nals [Wrana et al., 1992; Attisano et al., 1993]. Whereas type II receptor binds ligand on its own, type I receptor binds ligand when coexpressed with the corresponding type II receptor but not when expressed alone [Wrana et al., 1992; Attisano et al., 1993, 1996; Ebner et al., 1993; Franzén et al., 1993]. Furthermore, evidence suggests that type II receptors are constitutively active kinases [Wrana et al., 1992; Mathews and Vale, 1993] that associate with and phosphorylate type I receptors to form heteromeric complexes [Wrana et al., 1992, 1994], leading to their activation and signal propagation [Massague, 1998].

In the present study, we examined the possibility of inhibiting activin A-dependent erythroid differentiation in K562 cells using an activin type II truncated mutant lacking the cytoplasmic serine/threonine kinase domain. Since activin signals through heteromeric complexes of type I and II receptors, the mutant receptor would form nonfunctional complexes with endogenous receptors, hence blocking biological responses of activins. It was shown that when the truncated receptor lacking the kinase domain was expressed in K562 cells, erythroid differentiation induced by activin A was abrogated in these cells. These studies thus confirm that induction of erythroid differentiation by activin A is mediated through the K562 surface receptor. The implications on the mechanism of induction of erythroid differentiation are discussed.

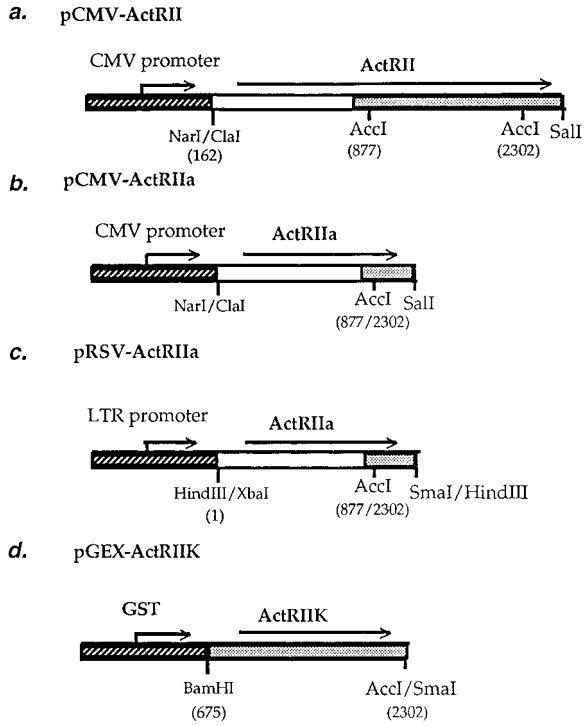

Fig. 1. Construction of expression vectors. **a:** pCMV-ActRII, the full-length activin type II receptor, ActRII cDNA, cloned into pCMV5 expression vector. **b:** pCMV-ActRIIa, construct of truncated type II receptor lacking kinase domain (positions 877–2302; accession No. M93415), which was derived from pCMV-ActRII by deletion of the region between two Acc I sites. **c:** pRSV-ActRIIa, construct of truncated type II receptor cloned into pRSV under the promoter control of RSV long terminal repeat (LTR). **d:** pGEX-ActRIIK, kinase domain of activin type II receptor cloned into pGEX-2T as a GST fusion protein. Blank areas represent extracellular and transmembrane domains; light shaded areas indicate cytoplasmic domain of ActRII, and the cross-hatched areas show the vector DNA.

MATERIALS AND METHODS

Preparations of Expression Vectors

Full-length cDNA of human activin type II receptor (ActRII) [Donaldson et al., 1992] and expression vector pCMV5 were kindly provided by Dr. L. Mathews (Salk Institute, La Jolla, CA) and Dr. M. Stinski (University of Iowa, Iowa City), respectively. K562 cell line was purchased from ATCC (Rockville, MD). Expression vectors containing full-length and truncated cDNA (pCMV-ActRII, pCMV-ActRIIa, and pRSV-ActRIIa in Fig. 1) were constructed and discussed in the Results section. For stable transfection of ActRIIa, expression vector pRSV [De Wet et al., 1987] was used in vector construction, using the *Xba*I site in the 5′ nontranslated region of ActRII and the *Hin*dIII site of pRSV (Fig. 1). For transfection with expression vectors, K562 cells were washed twice with buffer containing 20 mM Hepes, pH 7.5, 137 mM NaCl, 5 mM KCl, 0.67 mM glucose, and resuspended in 0.5 ml of the same buffer as 10^7 cells/ml; 40 μg of CsCl purified-plasmid DNA and 5 μg of pHSVneo (gift from Dr. J.A. DeCaprio, Dana-Farber Institute, Boston, MA) were added into the cell suspension. After 10 min at room temperature, electroporation was performed using the Bio-Rad Gene Pulser™ (Hercules, CA) at 960 mF, 220 V. G418 at 400 μg/ml was used for selection of Neo-resistant transfectants.

PCR Analysis

Approximately 1×10^5 cells were digested in 200 μl of 10 mg/ml protease K solution containing 100 mM Tris-Cl, pH 8.3, 500 mM KCl, 20 mM $MgCl_2$, 0.1% gelatin, 0.5% Nonidet P-40

(NP-40), 0.5% Tween-20, and incubated at 55°C for 2 h. The enzyme was then inactivated by heating at 95°C for 5 min and sample centrifuged for 5 min at 4°C. The supernatant was used as template for polymerase chain reaction (PCR) analysis. The PCR primers specific for type II activin receptor are: HActR1$^+$, 5′GAACTTC-CTCCGGATTCC3′, 140–157; HActR2$^+$, 5′AT-TTTTGTTGCTGCTGTGAGG3′, 475–492; and ActRm$^-$, 5′TAACTGGATTTGAAG3′, 550–564 (accession No. M93415) [Donaldson et al., 1992].

Iodination of activin A.

Human recombinant activin A (gift from Dr. R. Schwalls , Genentech, S. San Francisco, CA) was iodinated by chloramine T oxidation. A total of 5 μg of recombinant activin A and 1 mCi of free iodine-125 (NEN DuPont, Wilmington, DE) were used. The reaction was performed in 50 μl of 50 mM Na_2HPO_4/NaH_2PO_4 buffer, pH 7.5, containing 0.5 mg/ml of chloramine T, for 45 s and stopped by adding 10 μl of 1.0 mg/ml $Na_2S_2O_5$ and 20 μl of 0.5 mg/ml tyrosine. The iodinated activin A was separated from unreacted reagents by PD-10 desalting column (Amersham-Pharmacia, Piscataway, NJ) equilibrated in 50 mM Tris-Cl, pH 7.5, 1% bovine serum albumin (BSA), and a specific activity of 43,000 cpm/ng was obtained. The iodinated activin A was analyzed in 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Affinity Cross-linking Reaction

Approximately 10^7 cells were washed twice with culture medium without fetal calf serum (FCS). They were then resuspended in 0.5 ml of 25 mM HEPES buffered RPMI 1640 medium, and about 7.5×10^5 cpm (~0.6 pmol) of ^{125}I-activin A. For determination of nonspecific binding, 100-fold excess of cold unlabeled activin A was used in addition to the ^{125}I- activin A. Samples were incubated at 4°C for 3 h with constant rotation. Then, 1 ml of PBS was added before cells were pelleted. Cross-linking was done by resuspending the cells in 0.5 ml PBS and adding disuccinimidyl suberate (Sigma) to 0.5 mM. After incubating in ice for 30 min, the cross-linking reaction was terminated by addition of 1 ml of 50 mM Tris-Cl, pH 7.5, 150 mM NaCl. Finally, the cells were centrifuged and resuspended in 100 μl of 1% Triton X-100, 50 mM Tris-Cl, pH 7.5. After 1-h incubation in ice, the cell debris was removed by centrifugation and the supernatant was used for analysis in 8% SDS-PAGE.

Induction of Erythroid Differentiation in K562 Cells

Stock cultures of cells were grown in RPMI 1640 medium supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 50 IU/ml of penicillin, 50 μg/ml of streptomycin, and 10% FCS. The ability of transfected cells to be induced for erythroid differentiation was assayed in a 200-μl culture containing approximately 1×10^4 K562 cells in the presence of 25 ng/ml of activin A for 3 days [Yu et al., 1987]. The percentage of K562 cells containing hemoglobin was determined by benzidine staining [Orkin et al., 1975]. K562 cells induced by hemin was done in the same way as activin A, except that hemin was added to 25 μM in the cell culture.

Expression of the Kinase Domain As a Fusion Protein in *Escherichia coli*

The cytoplasmic domain of ActRII was first cut out at positions 877 and 2302 (accession No. M93415) of the ActRII cDNA [Donaldson et al., 1992] with AccI, then blunt-ended and subcloned into the expression vector pGEX-2T (Amersham Pharmacia) at the *Sma*I site to yield an intermediate vector (Fig. 1). The DNA sequence between positions at 675 and 1018 in ActRII, which contains the presumed ATP binding site [Donaldson et al., 1992], was obtained by PCR of ActRII cDNA with primers containing integrated *Bam*HI and *Afl*II sites at both ends. Then DNA was cloned into the *Bam*HI site of pGEX-2T and *Afl*II site at position 1018 of the intermediate vector, thus yielding pGEX-ActRIIk, which contains the cytoplasmic kinase domain of AcRII cDNA from 675–2302 (Fig. 1d)

The pGEX-ActRIIk was transformed into *E. coli* XL1-blue (Invitrogen, San Diego, CA) by electroporation [Dower et al., 1988] in Bio-Rad Gene Pulser™ with 2.2 kV, 220 mF. The expression of this GST fusion protein was done at 37°C with 1: 10 dilution of an overnight culture, and incubated for 2 h before adding isopropyl-β-D-thiogalactopyranoside to 0.1 mM for induction of 5 h as described [Smith et al., 1989].

Purification and Phosphorylation of the GST-ActRIIK Fusion Protein

Cells from expression culture were pelleted and resuspended in phosphate-buffered saline (PBS) containing 150 mM NaCl, 16 mM Na_2HPO_4, 4 mM NaH_2PO_4, pH 7.3, with 1/20 culture volume. The cell suspension was sonicated in the Cell Disruptor (Heat Systems-Ultrasonics, model W-225R) at maximum power for 2 min. After centrifugation, the soluble part of the lysed cells was discarded. Cell pellet containing the insoluble fusion protein was resuspended and washed in 2 M urea, 50 mM Tris-Cl buffer, pH 7.5, by centrifugation for 5 min at 10,000 rpm. After a second washing, the pellet was dissolved in 8 M urea in the same Tris buffer as above. Purification of the fusion protein was done by applying the proteins onto 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) [Laemmli, 1970]. GST-ActRIIK, which appeared as an approximately 66-kDa protein, was excised and electroeluted in 50 mM Tris/acetate buffer, pH 7.4, containing 0.1% SDS and 1 mM DTT for 3 h at 100 V [Leppard et al., 1983].

For phosphorylation of the fusion protein, samples were electrophoresed in SDS-polyacrylamide gels containing 10% glycerol, 5 mM DTT, and 0.5 mM $MgCl_2$ [Manser et al., 1992, 1994]. After blotting in 50 mM Tris base, 40 mM glycine, 10% methanol, 0.5 mM $MgCl_2$, 0.2% SDS, 0.2% Triton X-100 for 20 min at 15 mA onto PVDF membranes (Millipore, MA), these filters were treated with 6 M guanidine-Cl in buffer A (25 mM MES-NaOH, pH 6.5, 0.5 mM $MgCl_2$, 0.05 mM $ZnSO_4$, 0.05% Triton X-100). Membrane filters were agitated in this solution, which was then diluted with an equal volume of buffer A: this was repeated six times, and the filters were returned to "renaturing" PBS containing 1% BSA, 0.5 mM $MgCl_2$, 50 mM $ZnSO_4$, 0.1% Triton X-100 and 5 mM DTT at 4°C for 30 min [Manser et al., 1992]. Kinase reaction was carried out with incubation of the blotted membrane in 1 ml of 50 mM HEPES buffer, pH 7.0, containing 5 mM $MgCl_2$, 5 mM $MnCl_2$, 1 mM DTT, 0.05% Triton X-100 and 100 μCi ^{32}P-γ-ATP. After 30-min incubation at room temperature, the membranes were washed three times with PBS containing 6 M guanidine-Cl, 10% methanol, 5% acetic acid, and the results were analyzed by autoradiography.

RESULTS

Construction of Truncated Expression Vectors

The construction of expression vectors containing the full-length and truncated cDNA of activin type II receptors is illustrated in Figure 1. For expression of the full-length type II receptor cDNA, ActRII [Donaldson et al., 1992] was cleaved with restriction enzymes *Nar*I at position 162 and *Sal*I at the 3′ nontranslated region (accession No. M93415), and cloned into pCMV5 at *Cla*I and *Sal*I sites, to yield pCMV-ActRII (Fig. 1a). For expression of the truncated receptor lacking cytoplasmic kinase domain, restriction enzyme Acc I was used to delete the region corresponding to the cytoplasmic domain between 877 and 2302 of the full-length receptor cDNA in pCMV-ActRII, and then the remaining portion was religated to yield pCMV-ActRIIa (Fig. 1b). For stable transfection of the truncated receptor, ActRIIa, expression vector pRSV [De Wet et al., 1987] was used in vector construction, and the full-length receptor cDNA, ActRII, was first cleaved at its 5′ nontranslated region with *Xba*I and its 3′ region with *Sma*I, and then cloned into blunted-*Hin*dIII site of pRSV. This truncated form of type II activin receptor was designated as pRSV-ActRIIa (Fig. 1c). The junctions between vectors and inserted cDNAs in these constructs were all confirmed by DNA sequencing.

Integration of ActRIIa in Genome of K562 Cells

To demonstrate that the transfected K562 had integrated the ActRIIa cDNA into the genome of K562 cells, the following PCR of genomic DNA from both transfected and wild-type K562 cells was performed. The PCR primers used (Fig. 2B) were designed to distinguish between the endogenous genomic activin type II receptor DNA, which contains introns 2 and 3 [Stewart et al., 1986], and the exogenous transgene of the receptor cDNA in the transfectants. As shown in Figure 2A, after PCR amplication with $HActR2^+$ /$ActRm^-$ primers, a 250-bp band was observed in samples prepared from wild-type K562 cells as well as from ActRIIa transfected cells. This product is apparently derived from the endogenous type II receptor gene, as its size is the predicted length of genomic DNA with intron 3 (155 bp in size) (Fig. 2B). By contrast, a 95-bp band, corresponding to the expected size of an integrated

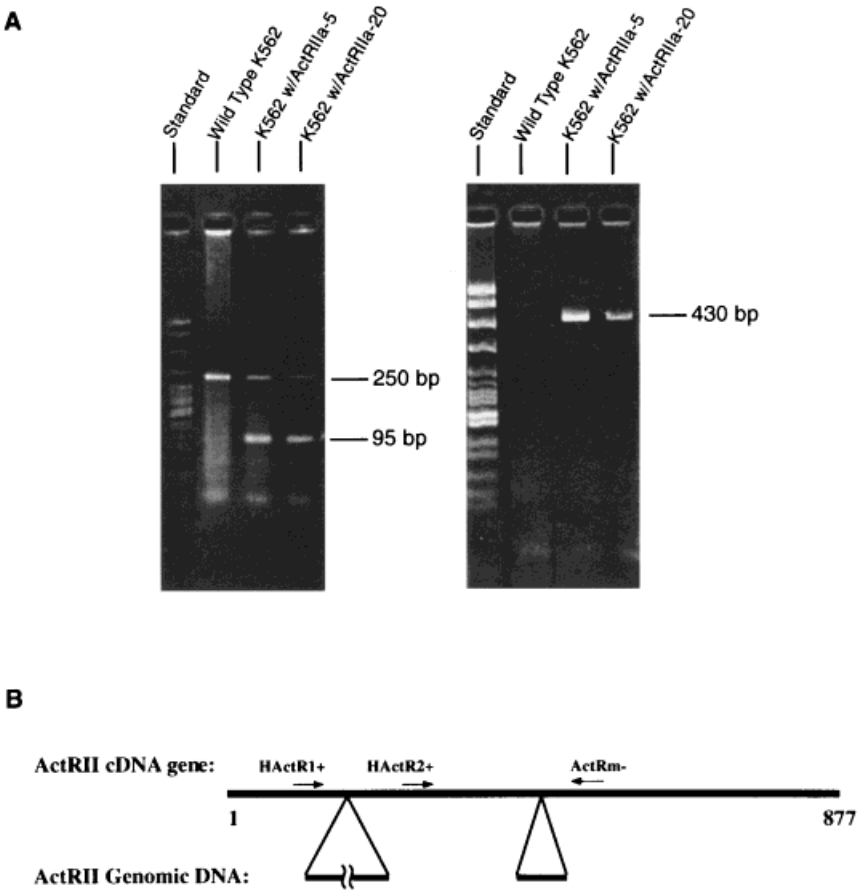

Fig. 2. Integration of ActRIIa in genomic DNA of the ActRIIa transfected K562 cells. **A:** Two sets of primers HActR1$^+$ (140–157)/ActRm$^-$ (550–564) and HActR2+ (475–493)/ActRm$^-$ (550–564) were used for performing PCR with DNA isolated from wild-type and pRSV-ActRIIa transfected K562 cells. On the left panel, primers of HActR2+/ActRm$^-$ were used. A PCR product of 250 bp shows the presence of endogenous type II receptor gene, while the 95-bp band is derived from the integrated ActRIIa in the pRSV-ActRIIa transfected cells (the clones 5 and 20). In samples from wild-type K562 cells, the 95-bp band was not found. On the right panel, when the HActR1$^+$/ActRm$^-$ primer set was used, a PCR product of 430 bp was found only in pRSV-ActRIIa transfected cells, but not in wild-type K562 cells. B: the locations of the PCR primers in cDNA and genomic DNA of type II activin receptor are shown with respect to introns 2 and 3 [Stewart et al., 1986].

type II cDNA in the cells, was observed only in samples from AcRIIa transfected K562 cells, but absent in samples from wild-type K562 cells. By contrast, the HActR1$^+$ /ActRm$^-$ primer set yielded a 430-bp PCR product in transfected cells, but no PCR product was observed in samples of wild-type K562 (Fig. 2A), as a large intron is present between the locations of these two primers in genomic DNA (intron 2 in Fig. 2B). These PCR Experiments thus confirmed the integration of pRSV-ActRIIa cDNA in the genome of the stable transfected K562 cells.

Cell Surface Expression of Truncated Type II Receptor

Affinity cross-linking with ^{125}I-activin A detected two distinct bands with molecular masses of approximately 90 and 70 kDa in wild-type K562 cells, corresponding to complexes with type II and type I receptors, respectively (Fig. 3, lane 3). In the presence of excess unlabeled activin A in the affinity labeling reaction, these two bands were not observed (Fig. 3, lane 4). In contrast, in the ActRIIa-transfected K562 cells, in addition to the aforementioned products, another ^{125}I-labeled band of approximately 60 kDa was observed (Fig. 3, lanes 1 and 2). Apparently, ^{125}I-labeled activin A bound specifically to its type I, type II receptors, as well as truncated type II receptors in these ActRIIa-transfected K562 cells. The sizes of these full-length and truncated receptors for activin agrees with the observations of similar affinity labeling and chemical cross-linking of COS-1 cells after transfection with various respective receptor constructs (data not shown) [see also Donaldson et al., 1992].

Effect of Truncated ActRIIa on Induction of Erythroid Differentiation

To investigate whether ActRIIa could actually act as a dominant negative mutant to block the activin A-dependent functional responses, we used human erythroleukemia cells that can be induced to differentiate. In agreement with

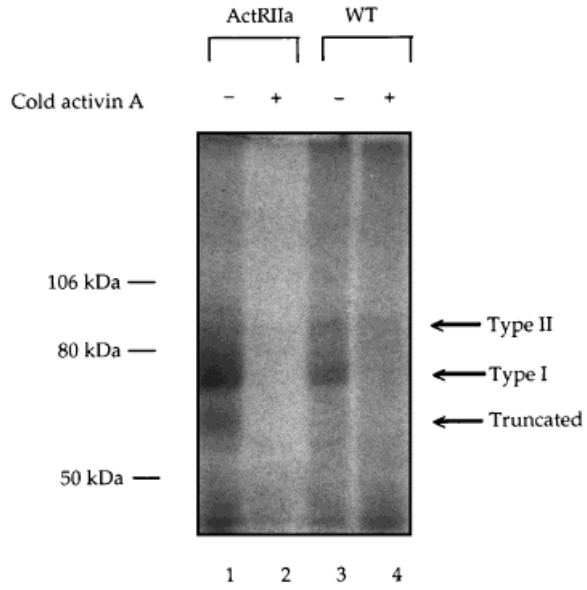

Fig. 3. Affinity cross-linking of 125I-labeled activin A to cell surface receptors in wild-type and ActRIIa transfected K562 cells. **Lanes 1,2**, ActRIIa transfected K562 cells cross-linked in the absence (−) or presence (+) of 100 ng/ml of unlabeled activin A, respectively; **lanes 3,4**, wild-type K562 cells (WT) in the absence (−) or presence (+) of unlabeled activin A, respectively. Specifically labeled bands were observed as approximately 70 kDa and 90 kDa corresponding to the wild-type type I and type II receptor-^{125}I-activin complexes, respectively. A band of approximately 60 kDa corresponding to truncated type II receptor-activin A complex is seen only in the ActRIIa transfected K562 cells. The affinity cross-linking procedure is described under Materials and Methods, and the ^{125}I-activin A bound proteins were resolved in 8% SDS-PAGE and autoradiographed. Molecular markers are indicated on the left.

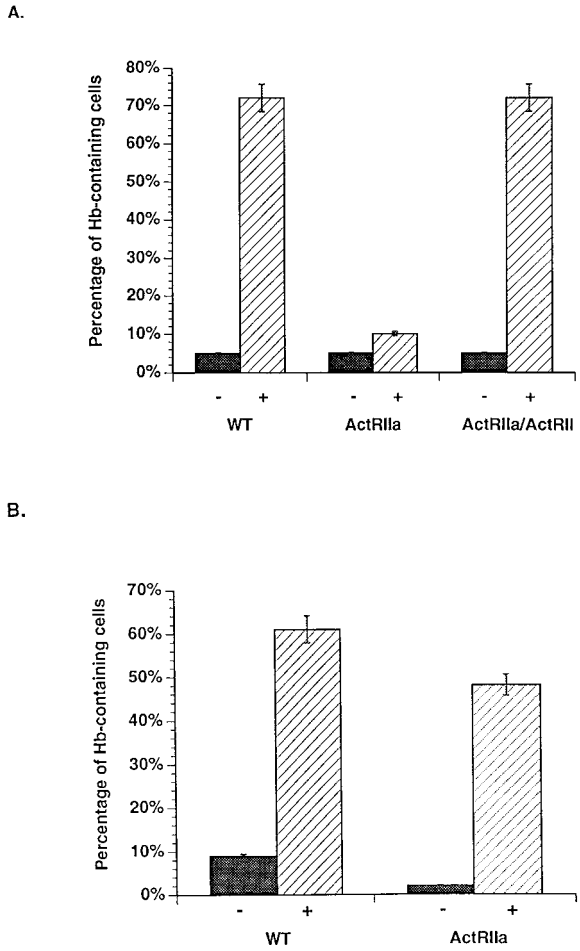

Fig. 4. Induction of erythroid differentiation in wild-type and ActRIIa transfected K562 cells. **A:** Wild-type K562 cells (WT) and K562 cells stably transfected with pRSV-ActRIIa (ActRIIa) were cultured in the presence (+) of 25 ng/ml of activin A. In addition, some of K562 cells containing ActRIIa were further transfected with full-length ActRII cDNA (ActRIIa/ActRII) and were similarly induced to differentiate with activin A. The controls were shown as the spontaneous induction without the addition of activin A into K562 cell cultures (−). **B:** Results of the chemical induction by 25 μM of hemin. The percentage of K562 cells containing hemoglobin was determined by benzidine staining [Orkin et al., 1975]. The results are mean values of triplicate determinations ±SE.

previous reports [Yu et al., 1987], treatment of wild-type K562 cells in culture for 3 days with human recombinant activin A caused induction of erythroid differentiation in cells to become hemoglobin-containing cells (approximately 70% in Fig. 4A). Induction of erythroid differentiation was similarly observed in mock transfected cells treated with activin A. In contrast, when various K562 stably transfected with pRSV-ActRIIa, which contained the truncated form of type II receptor lacking kinase domain (Fig. 1c), were treated with activin A, less than 10% of transfected cells were induced to differentiate and accumulate hemoglobin after 3-day culture (ActRIIa in Fig. 4A). In addition, increasing the amount of activin A used in cultures up to fivefold did not reverse the blockage of induction among K562 transfectants. These results indicate that the nonresponsiveness of ActRIIa transfected cells cannot be ascribed to the decrease in effective dosage of ligands as a result of expression of nonfunctional receptors on the cell surface. To examine whether the observed nonresponsiveness to ligands was caused by any nonspecific actions of transduction, we performed chemical induction of differentiation in transfected cells with hemin, which promotes K562 differentiation via pathway(s) different from that utilized via activin receptors. As shown in Figure 4B, K562 cells transfected with truncated receptor,

pRSV-ActRIIa, can be induced chemically by hemin just as wild-type cells. These data argue against nonspecific general actions caused by the expression of truncated receptors in the cells.

These results suggest that ActRIIa can act as a dominant negative mutant by forming a nonfunctional complex with endogenous activin receptors. To test the specificity of this dominant negative effect, we attempted to rescue the mutant phenotype by exogenous wild-type ActRII cDNA. As shown in Figure 4A (ActRIIa/ActRII cells), when wild-type ActRII was introduced into the K562 cells previously transfected with ActRIIa, the basal level of spontaneous induction remained the same. However, activin A treatment demonstrated the responsiveness of activin-dependent induction that was suppressed by ActRIIa in these ActRIIa transfected cells, indicating that the effect of ActRIIa is dominant negative. The high percent of hemoglobin-containing K562 cells in the ActRIIa/ActRII transfected samples is consistent with the reports that overexpression of wild-type activin receptors in K562 cells results in enhanced activin-induced erythroid differentiation [Lebrun and Vale, 1997].

Autophosphorylation of the Kinase Domain of ActRII

To confirm that the portion of activin type II receptor which was deleted in present studies contains kinase activities, a GST-fusion construct containing the deleted kinase domain was constructed (Fig. 1d) and expressed as fusion protein for the following experiments. After purification, renaturation, and in vitro phosphorylation as described under Materials and Methods, the autoradiograph of the membrane filters showed a distinct phosphorylated band at the size of 66 kDa, representing the GST-ActRIIK fusion protein in Figure 5. By contrast, no evidence of phosphorylation was observed with GST alone (Fig. 5). These experiments thus demonstrate that kinase domain of the type II receptor is capable of autophosphorylation and this activity is not regulated by its ligand binding because this fusion protein lacks the extracellular ligand binding domain of receptor.

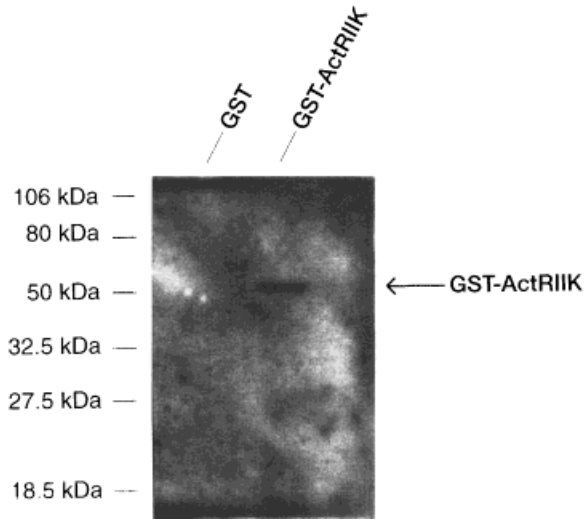

Fig. 5. Autophosphorylation of kinase domain of type II receptor. The kinase domain was expressed and purified as a GST fusion protein (GST-ActRIIK) as described under Materials and Methods. GST-ActRIIK was first subjected to 12% SDS-PAGE and transferred to PVDF membranes; kinase reaction was performed with the proteins blotted onto the filters. Autoradiograph of the filters shows that the GST-ActRIIK was phosphorylated (arrow), while the GST alone was not.

DISCUSSION

Two types of human activin receptors have been cloned; they are the type I receptor with apparent molecular weight of 55–60 kDa, and type II receptor with 65–75 kDa [Donaldson et al., 1992; Attisano et al., 1993]. Both receptors are highly glycosylated and share a variety of similarities with receptors for TGF-β [Mathews and Vale, 1993; Ying et al., 1997; Massague, 1998; Mathews, 1994]. Past studies have also shown that activins/TGF-βs bind their type I receptors only in the presence of type II receptors [Wrana et al., 1992; Attisano et al., 1993, 1996; Ebner et al., 1993; Franzén et al., 1993]. Evidence suggests that type I and II receptors are not only co-precipitated by antibodies [Mathews and Vale, 1993], but also form heteromeric complexes to generate signaling [Wrana et al., 1992, 1994; Massague, 1998; Attisano et al., 1993]. Therefore, expression of mutant receptors lacking some essential regulatory elements of the type II receptor would compete with endogenous wild-type receptor in complex formation, thus acting in a dominant negative fashion. It was also shown that overexpression of type I and type II receptors upon induction of K562 cells result in an increase in the hemoglobin content of the cells [Lebrun and Vale, 1997].

A mutant of activin type IIB receptor truncated at the cytoplasmic domain had been re-

ported to inhibit the normal development of the *Xenopus* embryos in vivo [Hemmati-Brivanlou et al., 1992]. It was also shown that the induction of the activin responsive 3TP-lux reporter construct in P19 embryonic carcinoma cells and their neuronal differentiation induced by activin were blocked by expression of a truncated activin receptor [De Winter et al., 1996]. In the present studies, truncated activin type II receptor lacking kinase domain was stably transfected into human K562 cells. It was found that in transfected K562 cells expressing the truncated type II receptor lacking the kinase domain, the induction of erythroid differentiation by activin A [Yu et al., 1987] was abrogated to a great extent. Furthermore, when exogenous full-length receptors were introduced into these ActRIIa transfected cells, activin induction rate was fully restored, in concordance with the dominant negative regulation of the truncated type II receptor in the transfectants. While the relative receptor numbers on the cells for ActRIIa versus ActRII, and the differentiation between binding to the homodimer versus heterodimer of these receptors had not been determined, present studies of these transfectants are consistent with the observations that overexpression of full-length activin receptors in K562 cells results in enhanced activin- induced erythroid differentiation [Lebrun and Vale, 1997]. It also remained to be determined whether the decrease in responsiveness toward activin A is due to the expression of truncated receptors on the cell surface, thus effectively competing for ligands. It was found, however, that increasing the amount of activin A added in the culture did not restore the ability of cells to be induced in these transfectants. Thus overexpression of truncated receptors did not result in ligand depletion; instead, the interaction between endogenous receptors and truncated receptors might lead to dominant negative inhibition for signal transduction.

The truncated cDNA was integrated into genome of the transfectants, as shown by polymerase chain reaction; and the expression of cell surface truncated receptor protein was shown by specific cross-linking with ^{125}I-labeled activin A in these transfectants. K562 cells express limited number of receptors (~600 per cell) [Campen and Vale, 1988]; therefore, transfected COS-1 cells that express more than 50,000 receptors per cell were used to confirm the identity of the expressed ActRII or ActRIIa constructs in vivo on the cell surface. Both COS-1 and K562 cells transfected with either ActRII or ActRIIa were found to yield similar size of full-length or truncated receptor/activin complexes after cross-linking with ^{125}I-activin A. Furthermore, because ^{125}I-labeled activin A bound to both full-length and truncated type II receptors, the absence of the cytoplasmic kinase domain in the truncated receptor did not affect the ligand binding of its extracellular domain and was also not involved in the heteromeric formation among receptors. It was found that the 90 and 70 kDa detected in affinity cross-linking of both wild-type and truncated ActRIIa transfected K562 cells correspond to type II and type I receptor complexes with their ligands. In addition, the ^{125}I-labeled band of approximately 60 kDa, which was observed only in the ActRIIa transfected K562 cells, corresponds to truncated type II receptor/activin A complex.

Evidence suggests that activin type II receptor not only associates with, but also phosphorylates type I receptor in cells [Wrana et al., 1992, 1994; Massague, 1998]. Full-length activin type II receptor had been shown to be phosphorylated in serine and threonine residues in vivo [Mathews and Vale, 1993]. However, autophosphorylation of GST-ActRIIK fusion protein in vitro demonstrates that phosphorylation of the receptor in vivo might not require ligand binding to the extracellular domain of receptor. This result agrees with previous observations that activin receptor purified from mouse embryonic carcinoma was phosphorylated in vitro, suggesting the state of constitutive phosphorylation for this receptor [Nakamura et al., 1992]. Therefore, in contrast to tyrosine kinase receptors where ligand binding induces dimerization and autophosphorylation of receptors [Schlessinger and Ullrich, 1992; Ullrich and Schlessinger, 1990], the surface receptors for activins and TGF-β with serine/threonine kinase domain may be autophosphorylated constitutively [Massague, 1998] and independent of ligand binding.

The present studies suggest that activin A exerts its effect on K562 through mediation with its receptors, because interference with receptor on cell surface inhibits the process of differentiation. Furthermore, these observations are in agreement with the report that expression of kinase-deficient activin receptors have an inhibitory effect on the activin-

dependent transcriptional response in activin-response cell lines [Tsuchida et al., 1995]. The effects of activin A on erythroid colony formation were thought to be indirect, requiring the presence of accessory cells [Yu et al., 1989; Broxmeyer et al., 1988]. Indeed, purified erythroid colony-forming units cultured in vitro with activin A did not increase numbers and size distributions of erythroid colonies [Shao et al., 1992a]. By contrast, in agreement with present findings, many studies had demonstrated that activin A can directly induce erythroleukemia cells into differentiation [Yu et al., 1987, 1989; Eto et al., 1987; Broxmeyer et al., 1988]. Incubation of purified erythroid progenitors and K562 cells with activin A was also found to lead to an increase in globin transcripts and hemoglobin accumulation [Frigon et al., 1992; Shao et al., 1992a]. Therefore, in addition to the indirect actions for promoting the proliferation of erythroid precursor cells, activin A may directly affect erythroid differentiation via interaction with receptors in the erythroid cells [Shao et al., 1992a; Yu et al., 1991]. In agreement with these suggestions, affinity labeling and chemical cross-linking of erythroid progenitors and K562 cells revealed two surface proteins (of 45–54 kDa) in these cells likely to be receptors for activin signaling [Shao et al., 1992a]. On the basis of these findings, it is suggested that erythroid differentiation caused by activin A is mediated directly through the surface activin receptor in erythroid progenitors.

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