

Inhibin Interferes with Activin Signaling at the Level of the Activin Receptor Complex in Chinese Hamster Ovary Cells*

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ABSTRACT

To gain more insight in the mechanism of action of inhibin, we studied the effect of inhibin on activin signaling in Chinese hamster ovary cells. Inhibin specifically counteracted activin-induced expression of a plasminogen activator inhibitor 1 promoter element (3TP) and of the *junB* gene, but was ineffective when the responses were induced by transforming growth factor- β . This indicates that inhibin acts only on the activin-specific part of these signaling cascades. Using a constitutively active activin type IB receptor we determined whether inhibin acted at the level of the activin-receptor complex or downstream of it. The mutant activin receptor stimulated the ex-

pression of the 3TP promoter in the absence of activin. This stimulation was insensitive to inhibin, indicating that inhibin acts exclusively at or upstream of this activin type I receptor. In addition, competition studies using labeled activin showed that inhibin displaced activin from the activin type II receptors, especially from the activin type IIB receptor, but not from the type I receptors. In conclusion, these data show that in Chinese hamster ovary cells inhibin acts directly at the activin receptor complex, most likely through displacement of activin from the activin type II receptor. (*Endocrinology* 138: 2928–2936, 1997)

INHIBIN AND activin were originally defined as gonadal hormones regulating the release of FSH from the anterior pituitary gland (1–3). Today, these factors are known to play additional local roles in the gonads (4) as well as in extragonadal tissues, such as in erythrocyte differentiation (5), mesoderm induction (6, 7), apoptosis of liver parenchyme (8), and extracellular matrix formation (9). Further, they modulate the growth of a number of different cell types and cell lines (10–13). In many cell types, inhibin counteracts responses to activin; in some cases, however, inhibin is ineffective (11, 14, 15).

Activin and inhibin are both members of the transforming growth factor- β (TGF β) superfamily of growth and differentiation factors. Members of this family consist of two identical or highly homologous subunits, linked by one disulfide bridge (16, 17). Activin is consistent with this model; it is a dimer of two inhibin β -subunits. Inhibin is the only exception in this family, as it consists of an inhibin β -subunit, which is also present in activin, linked to a distantly related inhibin α -subunit. Furthermore, no homodimers of the α -subunit of inhibin have been described.

Members of the TGF β superfamily exert their actions

through combinations of type I (55 kDa) and type II (68 kDa) receptors. Both receptors are characterized by a small extracellular ligand-binding domain, a single transmembrane domain, and an intracellular Ser/Thr-specific kinase domain. For both TGF β and activin, the type II receptor is a constitutively active kinase that has high affinity for the ligand. This ligand type II receptor complex subsequently interacts with the type I receptor (18, 19). After association, the type I receptor is phosphorylated by the kinase domain of the type II receptor in its juxta-membrane region, also known as the GS box (18–20). The phosphorylation of the GS box apparently leads to activation of the type I receptor, resulting in stimulation of downstream pathways.

Two activin type II receptors (ActRIIA and ActRIIB) and two activin type I receptors (ActRIA and ActRIB, also known as ALK-2 and ALK-4, respectively) are known (21–24). However, inhibin receptors have not been identified to date, and the mechanism of action of inhibin has not been clarified. It might be that inhibin signals through its own type I and type II receptors. Alternatively, the special position of inhibin in the TGF β superfamily may indicate that its signaling mechanism is different, as is also suggested by the fact that inhibin appears to interfere specifically with activin signaling. We investigated whether inhibin blocks activin signal transduction downstream of the activin-receptor complex or interferes with activin signaling at the level of the activin receptor as suggested earlier (13, 25). As a model, the Chinese hamster ovary (CHO) cell line K1 was used; its cell growth is sensitive to both activin and inhibin (11). In these cells the interference of inhibin with activin-induced immediate early responses

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and the effect of inhibin on the activin receptor complex were studied. This showed that inhibin acts directly at the level of the activin-receptor complex in CHO cells. In addition, our data indicate that inhibin can interfere with activin signaling through displacement of activin from the activin type II receptor.

Materials and Methods

DNA constructs

The mouse ActRIA, -IB, -IIA, -IIB₂, and -IIB₄ clones used were previously described (21, 24, 26) (Miyazono, K., unpublished observations). The ActRIIs used were not modified, whereas the ActRIIA and the ActRIIBs were extended by the addition of an HA tag (27) and a KT₃ tag (28), respectively, at their 3'-end as previously described (29). The expression of all constructs was under control of the human β -actin promoter (30). The p3TP-Lux construct used was described previously (31). The p5T-Lux construct was obtained by replacing the chloramphenicol acetyltransferase (CAT) gene in pTRE-TATA-CAT (32) using *Xho*I and *Syl*I with the luciferase gene of the pGL2-basic-Lux vector (Promega Corp., Madison, WI). The human ActRIIB (23) was used as a template for PCR mutagenesis to introduce a GAT (Asp) codon instead of an ACC (Thr) codon at position 206 from the start codon. Both wild-type and mutated receptors were introduced in the expression plasmid pcDNA3 (Invitrogen, Leek, The Netherlands).

Transfection and luciferase assay

CHO K1 cells were maintained in DMEM-Ham's F-12 (DF; Life Technologies, Gaithersburg, MD) supplemented with 10% FCS (SEBAK, Aidenbach, Germany) and cultured at 37 C in 5% CO₂ in air. Cells were plated before transfection in 12-well plates (Costar, Cambridge, MA) at a concentration of 4×10^4 cells/well. The next day the cells were transiently transfected with either p3TP-Lux or p5T-Lux (0.5 μ g/well) using the calcium phosphate transfection method (33). In case of co-transfection with activin receptor expression plasmid, up to 1.0 μ g of this plasmid was used per well. To correct for transfection efficiencies, the β -galactosidase expression plasmid pCH110 (0.5 μ g/well) (34) was added. At 50% confluence, cells were deprived of serum and cultured in DF containing 0.1% BSA for at least 8 h. Subsequently, the cells were incubated with vehicle, human recombinant activin A (Innogenetics, Ghent, Belgium), human recombinant inhibin A (Genentech, South San Francisco, CA), TGF β (Sanbio, Breda, The Netherlands), β -phorbol 12-myristate 13-acetate (PMA; Sigma Chemical Co., St. Louis, MO), or steroid-free bovine ovarian follicular fluid as indicated. After 16 h, the cells were lysed, and luciferase and β -galactosidase activities were measured (35, 36).

Northern blot analysis of *junB* messenger RNA (mRNA)

Serum-deprived cells were treated as described and harvested 0, 0.5, 1.5, or 6 h after the addition of activin or activin and inhibin. Total RNA was extracted by the TRIzol procedure (Life Technologies), and 20 μ g of this were fractionated on a denaturing agarose gel and then transferred to a nylon membrane (Hybond N⁺, Amersham, 's-Hertogenbosch, The Netherlands) (37). The *junB* mRNA was detected using mouse *junB* complementary DNA (cDNA; p465.20) as a probe (38). Hybridization with a glyceraldehyde-3-phosphate dehydrogenase cDNA served as a measure of the amount of RNA applied to each lane. The labeled probe bound to the blot was quantified using a PhosphorImager and the ImageQuant software package (version 3.3, Molecular Dynamics/B&L Systems, Zoetermeer, The Netherlands).

Reverse transcriptase-PCR (RT-PCR)

RT-PCR was performed on total RNA of CHO cells and K562 cells as previously described (39) using the oligonucleotide primers described below. As a control, PCR was performed on cDNA clones of the mouse ActRIIA, ActRIIB₂, and ActRIIB₄ described above. For ActRIIA, primers derived from the rat cDNA sequence (40) were used for the amplification (forward primer, 5'-CAGGGAAGCTG GATATCTAGA GAGAAGTTC-

3'; reverse primer, 5'-TGGTCTGGG TCTCGAGTAG GAACAAG-TAC-3'); for ActRIIB, primers derived from the human ActRIIB (41) were used (forward primer, 5'-CGAATTCGGC TGTCGCCCAT TGGAGGC-3'; reverse primer, 5'-TGTAAGCTTG TGGCCCTCAC CACGACCC-3'). The ActRIIA primers amplify a fragment of 685 bp; the ActRIIB primers amplify a 790-bp fragment for the ActRIIB₂ and a 766-bp fragment for the ActRIIB₄. The PCR reaction consisted of 40 cycles of denaturation at 94 C for 1 min, primer annealing at 50 C for 2 min, and subsequent extension reaction at 72 C for 2 min using 0.2 U SuperTaq (HT/Biotechnology, Cambridge, UK). Reaction products were analyzed by agarose (2%) gel electrophoresis and visualized by ethidium bromide staining.

Cross-linking

COS-1 cells were maintained in DF supplemented with 7.5% FCS and cultured at 37 C in 5% CO₂ in air. Wild-type CHO cells or activin receptor-transfected COS cells ($\sim 2-4 \times 10^6$ cells) were preincubated for 30 min at 4 C without additions or with unlabeled activin A or inhibin A followed by an incubation of 2 h at 4 C with 10 ng [¹²⁵I]activin A (180 pM; $\sim 750,000$ cpm) in 2 ml DF. Activin A was iodinated using chloramine-T as previously described (29). After the incubation, the cells were washed twice with HEPES-buffered saline. Bound activin was cross-linked to its receptor with 1 mM bis-sulfosuccinimidyl suberate (BS³, Pierce Chemical Co., Rockford, IL) as described previously (29). Subsequently, the cells were lysed, and the cross-linked complexes were purified. For CHO cells, this was achieved by incubating the extracts with wheat-germ agglutinin agarose overnight; for activin receptor-transfected COS-1 cells, the complexes were first incubated overnight with specific antibodies to either the HA or the KT₃ tag present on the C-terminus of the ActRII. The resulting complexes were subsequently isolated by an incubation with either protein A- or protein G-Sepharose, as previously described (29), and subsequently separated on a reducing SDS-PAGE followed by autoradiography. The amount of iodinated activin cross-linked to either activin receptor was quantified using a PhosphorImager and the ImageQuant software package.

Results

Inhibin blocks expression of immediate early genes induced by activin

To study the mechanism of action of inhibin in CHO cells, we initially determined whether inhibin was able to affect activin-induced immediate early expression of the *junB* gene and the commonly-used artificial 3TP-Lux reporter gene. The 3TP-Lux reporter construct contains a TGF β /activin responsive region of the plasminogen activator inhibitor 1 (PAI-1) promoter and three 12-O-tetradecanoylphorbol acetate (TPA)-responsive elements (TREs) (31). Activin (0.9 nM; ED₅₀ = 180 pM) stimulated the luciferase activity in CHO cells transfected with the 3TP-Lux reporter construct 6- to 8-fold. The luciferase activity was already detectable after 4 h, but was maximal between 16-24 h after the start of the incubation (not shown). Inhibin on its own did not elicit a response, but it reduced the response to activin dose-dependently (Fig. 1, A and C). To investigate whether this activin-induced response was specifically affected by inhibin, we also studied the effect of inhibin on TGF β -induced 3TP reporter activity. TGF β was chosen because it is known to induce similar responses as activin, including 3TP reporter activity, in a number of cell types (11, 42, 43). TGF β (150 pM; ED₅₀ = 25 pM) stimulated luciferase activity in the 3TP-Lux construct 8- to 10-fold (Fig. 1A; dose-response curves not shown). This TGF β -induced luciferase activity, however, was insensitive to inhibition by inhibin (Fig. 1A). Even a relatively high doses of inhibin (2 nM) was completely ineffective (not shown). Activin and TGF β both act through either one of the two

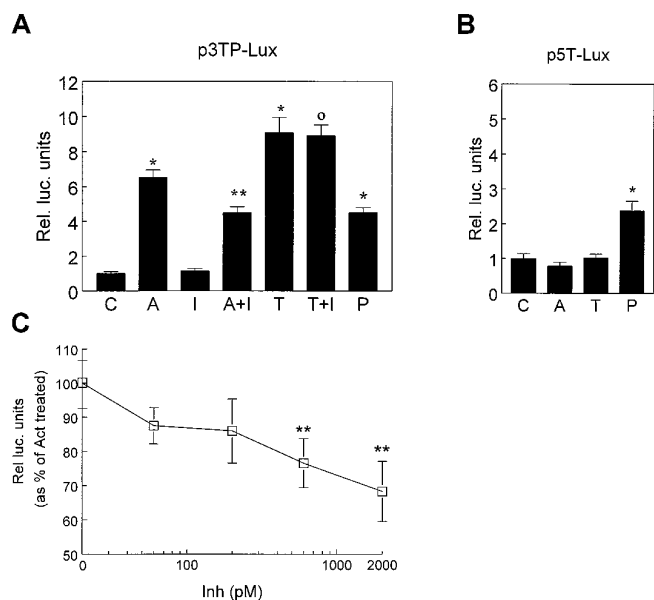


FIG. 1. Inhibin inhibits activin-dependent 3TP promoter activity. Relative luciferase activity was determined in CHO cells that were transiently transfected with either p3TP-Lux (A and C) or p5T-Lux (B). Transfected cells deprived of serum were stimulated for 16 h with vehicle (C), activin (A; 900 pM), TGF β (T; 150 pM), inhibin (I; 900 pM), or PMA (P; 100 nM), alone or, when indicated, in combination with inhibin (900 pM). For the dose-response curve for inhibin (C), cells were treated with activin (900 pM) in combination with the indicated concentrations of inhibin. Luciferase activity was measured in cell lysates and is plotted as the mean \pm SEM (n = 6). Data were subjected to one-way ANOVA according to Scheffe. *, Significantly different from control cells ($P < 0.05$); **, significantly different from control and activin-treated cells ($P < 0.05$); ^o, significantly different from control, but not different from TGF β -treated cells ($P < 0.05$). The results of one experiment of three with similar results are shown.

different response elements in the 3TP promoter. Therefore, we studied the effect of activin, TGF β , and the protein kinase C activator, PMA, on a reporter construct containing just TREs, p5T-Lux. PMA (50 nM) stimulated the reporter activity of this construct in CHO cells 3-fold, whereas activin (1 nM) and TGF β (250 pM) had no effect (Fig. 1B). In addition, we found that the effects of TGF β and PMA on the 3TP-Lux construct were additive (not shown). Thus, the transcription from the 3TP promoter induced by both activin and TGF β is likely to be exclusively derived from the PAI-1 promoter element in the 3TP-Lux construct. As inhibin only interferes with the activin-induced 3TP promoter activation, inhibin exerts its effect on a part of the activin signaling cascade that is not in common with that of TGF β .

In addition, we studied whether expression of an unrelated activin-inducible immediate early gene, *junB*, was also sensitive to inhibin. To this end, CHO cells were treated with activin in the absence or presence of inhibin. Activin induced *junB* mRNA levels 4-fold within 30 min, but the response rapidly declined thereafter (Fig. 2). Inhibin reduced the activin-dependent *junB* expression significantly ($P < 0.005$, by Mann-Whitney U test). Equimolar amounts of inhibin reduced activin-induced *junB* expression by $28 \pm 4.1\%$ and reduced activin-induced 3TP luciferase activity by $27 \pm 7\%$. This indicates that both immediately early responses to ac-

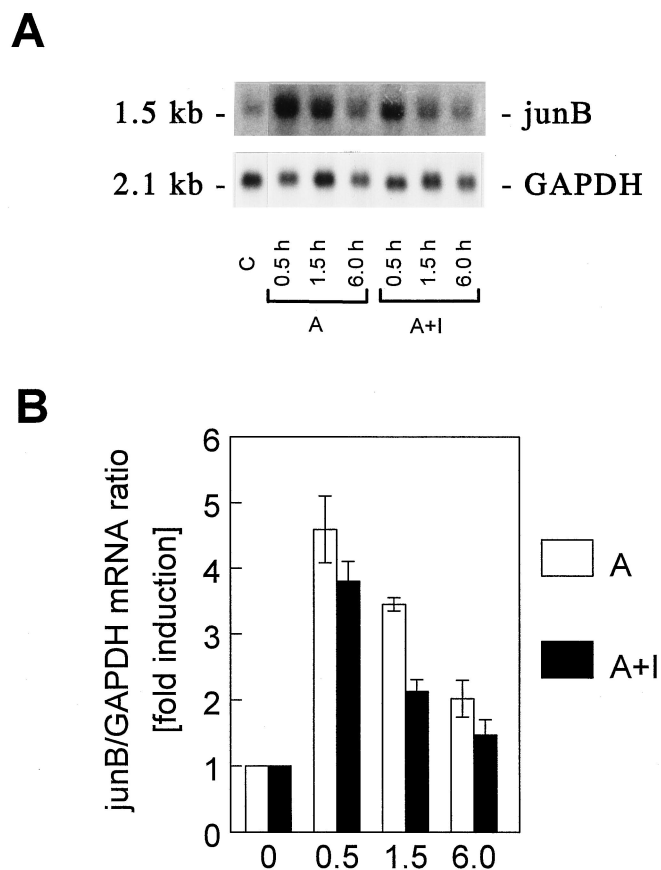


FIG. 2. Induction of *junB* mRNA expression in CHO cells by activin and inhibin. The *junB* mRNA expression was determined by Northern blot analysis in CHO cells treated for 0, 0.5, 1.5, and 6 h with activin (900 pM) or with activin in combination with inhibin (800 pM). A, Autoradiograph of the Northern blot. The size of the mRNA is indicated on the left. B, Quantification (see Materials and Methods) of *junB* mRNA levels. The *junB* mRNA/glyceraldehyde-3-phosphate dehydrogenase mRNA levels are plotted against the duration of the incubation. The mean \pm SD of two independent experiments are shown. The *junB* mRNA time curve of cells treated with activin is significantly different from that of cells treated with activin and inhibin (by Mann-Whitney U test, $P < 0.005$).

tivin are equally sensitive to inhibin. On a molar basis, however, inhibin is less effective than activin.

Inhibin competes with activin at the activin-receptor complex in COS-1 and CHO cells

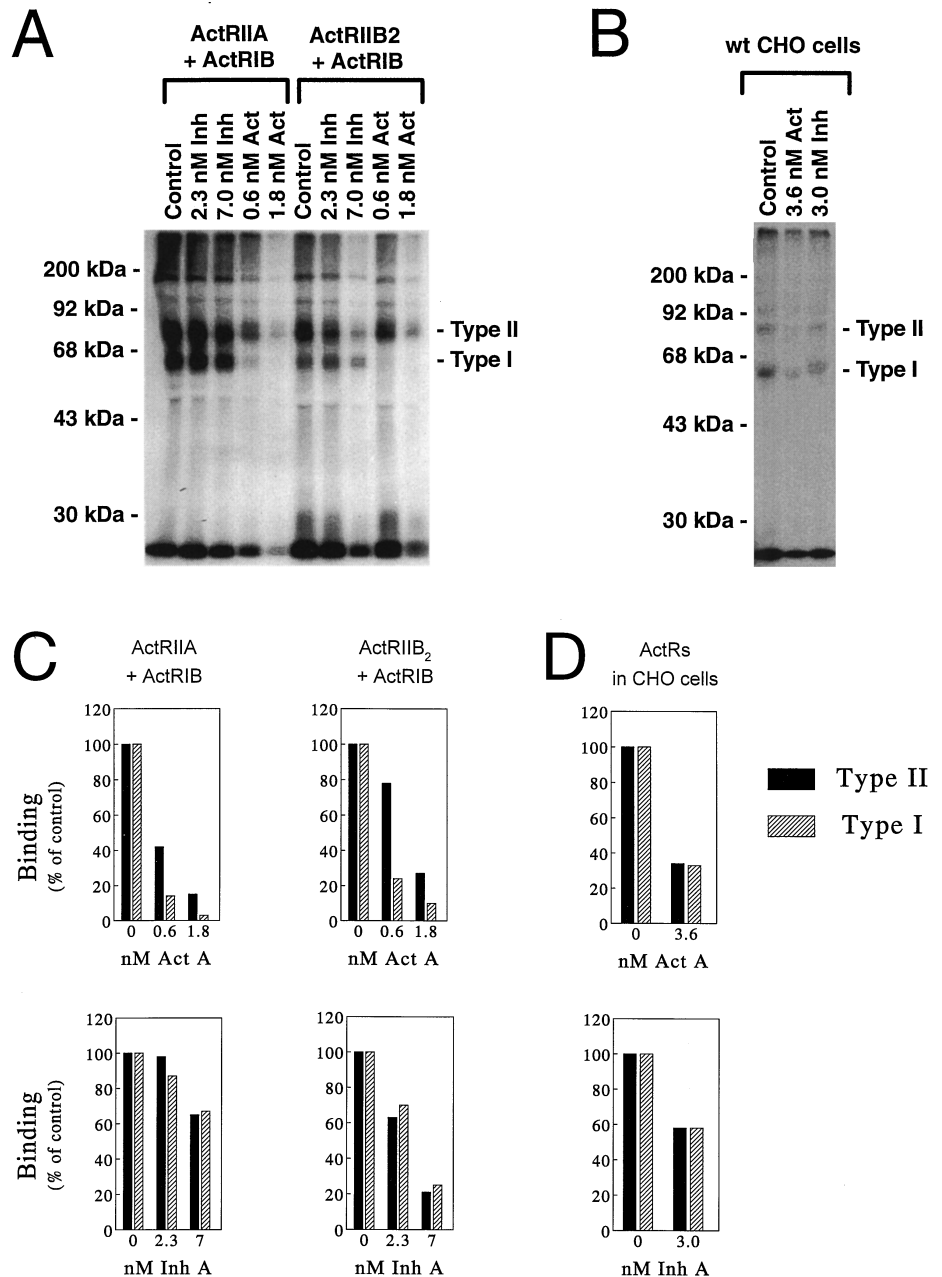
Experiments reported by Carcamo and co-workers (42) suggested that the pathways for 3TP promoter activation, PAI-1 expression, and growth inhibition downstream of the type I receptors for TGF β and activin are identical. Because inhibin did not interfere with the effects of TGF β , this implies that the activin-receptor complex itself is the primary target for inhibin action in CHO cells. We, therefore, studied whether inhibin was able to displace activin from the activin-receptor complex. Previous work showed that inhibin, although with low potency, is able to displace activin from the ActRIIs (21, 24, 25). These experiments, however, were performed on cells expressing type II receptors but no type I receptors. We included the two ActRIIs in our analysis be-

cause they are an essential part of the activin signaling complex (18, 19) and could also be targets for inhibin or required for inhibin action. Using transient transfection, different sets of ActRI and ActRII cDNAs were introduced into COS-1 cells. COS-1 cells were used because these cells do not express endogenous activin receptors that might interfere with the assay (21). The transfected cells were incubated with unlabeled activin (0.6 or 1.8 nM), unlabeled inhibin (2.3 or 7 nM), or vehicle to allow ligand binding. Subsequently, iodinated activin (0.18 nM) was added, and the incubation was continued for 2 h. Bound hormone was cross-linked to the receptors, immunoprecipitated with an antibody to the tagged ActRII, and subsequently analyzed by SDS-PAGE under reducing conditions. The results of ActRIB in combi-

nation with ActRIIA or with the most common splice variant of ActRIIB, ActRIIB₂ (24, 41), are shown (Fig. 3A). Experiments with another splice variant of ActRIIB, ActRIIB₄, were also performed. This splice variant differs from the ActRIIB₂ in a small part of the extracellular domain that affects the affinity for activin (24). The results obtained with ActRIIB₄ were comparable to those obtained with ActRIIB₂ (not shown). In addition, ActRIA was tested in combination with all three type II receptors. The results obtained with this receptor were identical to those with ActRIB (not shown).

Iodinated activin subunits were predominantly cross-linked to the ActRI and ActRII, resulting in complexes of 65 and 85 kDa in size, respectively (Fig. 3A, control lanes). In addition, some minor larger complexes (100–160 kDa)

FIG. 3. Inhibin competes with activin for binding to the activin-receptor complex. Wild-type CHO cells (B and D) or COS-1 cells, cotransfected with ActRIB and the indicated ActRII cDNA expression vectors (A and C), were affinity labeled by incubation with iodinated activin alone (control) or in the presence of unlabeled activin (Act) or inhibin (Inh). After cross-linking with bis-sulfosuccinimidyl suberate, the cells were lysed, and the receptor complexes were immunoprecipitated using antibodies against the tags of the different ActRIIs or, in case of CHO cells, with wheat-germ agglutinin agarose. Precipitates were subjected to SDS-PAGE under reducing conditions, followed by autoradiography. The autoradiographs are shown in A and B. The amount of labeled activin cross-linked to the ActRI and ActRII was quantified using a PhosphorImager and plotted against the dose of unlabeled activin or inhibin added (C and D). The results of a representative experiment are shown; essentially similar results were obtained in three independent experiments.



were observed, presumably consisting of multiple activin subunits and/or activin receptors cross-linked to each other (13).

Preincubation with unlabeled activin resulted in a gradual decrease in the amount of labeled activin cross-linked to the ActRI and ActRII (Fig. 3A). The ratio of the amount of labeled activin cross-linked to the ActRI over that cross-linked to ActRII decreased (to $35 \pm 9\%$ and $40\% \pm 6\%$ for ActRIIA and ActRIIB₂, respectively) relative to the ratio in the absence of competitor ($P < 0.05$, by Student's *t* test; $n = 3$; Fig. 3C). In the presence of inhibin, the amounts of labeled activin cross-linked to both ActRII and ActRI also decreased (Fig. 3A). However, the ratio between the amount of labeled activin cross-linked to the ActRI and that cross-linked to ActRII did not change significantly after the addition of inhibin ($99 \pm 6\%$ and $107\% \pm 20\%$ for ActRIIA and ActRIIB₂, respectively) compared to that in the absence of competitor (Fig. 3C). This difference between activin and inhibin was observed for all ActRI and ActRII combinations tested. In the presence of unlabeled inhibin, labeled activin was cross-linked more efficiently to ActRIIA than to ActRIIB₂; inhibin was approximately 15 times less potent than activin in competing for labeled activin bound to ActRIIA, whereas this difference for ActRIIB₂ was only 2.5. This difference was independent of the type I receptor (IA or IB) transfected into COS-1 cells. This set of experiments indicates that ActRIIs, but not ActRIs, can be targets for inhibin and that inhibin preferentially interacts with ActRIIBs.

In addition, we performed similar displacement studies with the endogenous activin receptors of CHO cells that were incubated with labeled activin (0.18 nM) alone or in the presence of unlabeled activin (3.6 nM) or inhibin (3.0 nM). After cross-linking, the complexes were purified by binding to wheat-germ agglutinin agarose beads and analyzed on SDS-PAGE (Fig. 3B). Similar to the results of the COS-1 cell experiments, labeled activin was predominantly cross-linked to a type I and a type II receptor and unlabeled activin and inhibin reduced the amount of labeled activin cross-linked to both type I and type II receptors (Fig. 3B). However, unlike the findings in COS-1 cells, unlabeled activin did not affect the ratio of labeled activin cross-linked to ActRI and ActRII (Fig. 3, B and D). Inhibin was 2–3 times less potent than unlabeled activin in displacing labeled activin from the endogenous ActRII in CHO cells (Fig. 3D).

To identify the ActRIIs involved in this binding, we analyzed which of the known ActRIIs are expressed in CHO cells. Expression levels of ActRIIs were too low to be detected on a Northern blot. Therefore, we identified the receptors by PCR using primers specific for either ActRIIA (Fig. 4A) or ActRIIB (Fig. 4B). Both receptors were detected in CHO cells; the only ActRIIB splice variant present was ActRIIB₂. Another inhibin-responsive cell line, K562, which predominantly expresses ActRIIB₂, as determined by ribonuclease protection (van Schaik, R. H. N., unpublished results) was included in this analysis as a control. In these cells, ActRIIB₂ could be amplified to a prominent band (Fig. 4B, lane 7), whereas no ActRIIA could be detected in these cells.

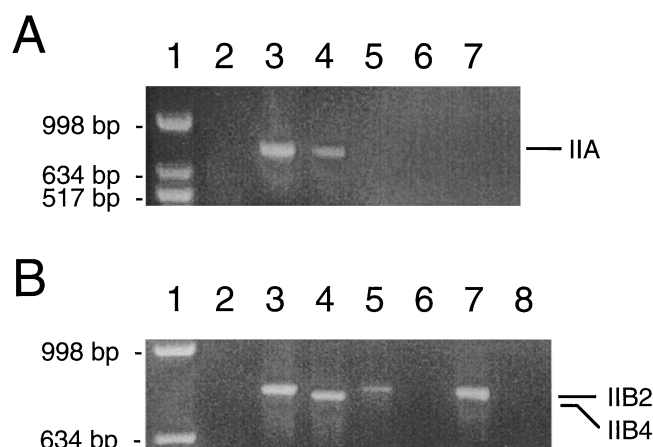


FIG. 4. ActRIIA and ActRIIB₂ are expressed in CHO cells. RT-PCR of total RNA was performed to identify ActRIIA (A) and ActRIIB (B) in CHO and K562 cells. The PCR products were subsequently analyzed on a 2% agarose gel. A, Detection of ActRIIA mRNA: pBR322 digested with *Hinf*I and *Eco*RI (lane 1); control PCR (lane 2); PCR of cDNA clone of mouse ActRIIA (lane 3); RT-PCR of total RNA of CHO cells (lane 4, with RT; lane 5, without RT); RT-PCR of total RNA of K562 cells (lane 6, with RT; lane 7, without RT). B, Detection of ActRIIB mRNA: pBR322 digested with *Hinf*I and *Eco*RI (lane 1); PCR control (lane 2); PCR of cDNA clone of mouse ActRIIB₂ (lane 3) and of mouse ActRIIB₄ (lane 4); RT-PCR of total RNA of CHO cells (lane 5, with RT; lane 6, without RT); RT-PCR of total RNA of K562 cells (lane 7, with RT; lane 8, without RT). The results of one representative experiment of two are shown.

Inhibin is unable to block the response from a constitutively active ActRIB

To exclude a role for inhibin in the activin signal transduction pathway downstream of the receptor complex, the ability of inhibin to inhibit the induction of 3TP-Lux reporter activity by a constitutively active ActRIB receptor was investigated. In analogy with the constitutively active TGF β type I receptor (TGF β RI) (44), we replaced threonine 206 located between the GS box and the kinase domain by aspartate in the ActRIB (T206D). The ActRIB was chosen because ActRIB and not ActRIA mediates PAI-1 expression and growth inhibition by activin in Mv1Lu cells (42), and ActRIB mediates 3TP promoter activation by activin in CHO cells (45). To ascertain the constitutive activity of this mutant receptor, we compared 3TP promoter activation in the absence of activin in CHO cells transfected with increasing amounts of wild-type or mutant ActRIB expression plasmid (Fig. 5A). In cells transfected with the wild-type receptor construct, luciferase activity was low and did not depend on the amount of expression plasmid. In contrast, the luciferase activity considerably increased with increasing amounts of expression plasmid when the mutant ActRIB was introduced. This indicates that the replacement of threonine 206 by aspartate leads to the activation of the ActRIB in the absence of activin, as was also recently shown by Attisano *et al.* (18). The constitutively active receptor did not activate the 5T-Lux construct containing only TREs (Fig. 5B), showing that 3TP promoter activation by this mutant receptor is promoter specific and is not due to aspecific changes due to, for example, overexpression of this receptor. Subsequently, CHO cells were transfected with either wild-type ActRIB or

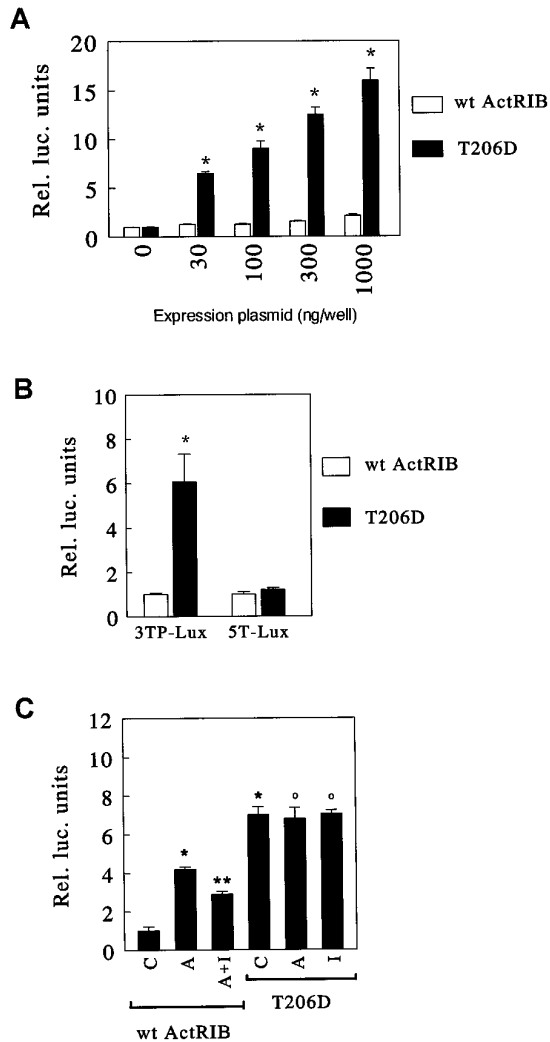


FIG. 5. Inhibin acts upstream of the activin type IB receptor. A, Mutant ActRIB (T206D) is constitutively active. The luciferase activity of transfected CHO cells was determined as described in Fig. 1. CHO cells were cotransfected with the p3TP-Lux reporter plasmid, a β -galactosidase expression construct pCH110, and increasing amounts (nanograms per well) of wild-type or mutant ActRIB receptor (T206D) expression vector. β -Galactosidase was measured to correct for transfection efficiency. B, ActRIB activates the PAI-1 promoter element in the 3TP promoter and not the TREs. CHO cells were cotransfected with the p3TP-Lux or the p5T-Lux reporter plasmid in combination with wild-type receptor (open bar) or ActRIB-T206D (closed bar), and luciferase activity was determined as described above. C, Inhibin does not inhibit ActRIB(T206D)-induced 3TP promoter activation. Cells were cotransfected with p3TP-Lux and pCH110 in combination with either wild-type or mutant ActRIB (T206D; 100 ng/well). Wild-type ActRIB-transfected cells were incubated with vehicle (C), activin (A; 900 pM) alone, or activin in combination with inhibin (I; 900 pM); ActRIB-T206D-transfected cells were incubated with similar doses of activin and inhibin. Relative luciferase activity was determined in cell lysates and is plotted as the mean \pm SEM (n = 6). Data were subjected to one-way ANOVA according to Scheffe. *, Significantly different from wild-type ActRIB-transfected control cells ($P < 0.05$); **, significantly different from activin-treated wild-type ActRIB-transfected control cells ($P < 0.05$); \circ , not different from ActRIB-T206D-transfected control cells ($P > 0.05$). The results of one experiment of three with similar results are shown.

ActRIB(T206D), and 3TP promoter activation was studied in response to activin and/or inhibin (Fig. 5C). CHO cells transfected with the wild-type ActRIB responded to activin with a 4- to 5-fold increase in luciferase activity, and this activin-dependent luciferase activity was sensitive to inhibin, similar to that in wild-type CHO cells. In CHO cells transfected with the mutant ActRIB, basal luciferase activity was increased about 10-fold compared to that in cells transfected with the wild-type ActRIB. Activin did not further increase luciferase activity in these cells, suggesting that this mutant receptor was already fully active in the absence of activin. More importantly, the addition of inhibin (0.8 nM) was also without effect (Fig. 5C). Even a high concentration of inhibin (2 nM) or bovine follicular fluid containing 10 nM bioactive inhibin did not decrease 3TP promoter activity (not shown). This experiment shows that inhibin is unable to block 3TP promoter activation induced by activin downstream of the ActRIB in CHO cells.

Discussion

Inhibin is an endocrine and paracrine inhibitor of locally induced activin responses in both the pituitary gland (2, 3) and the gonads (4). However, the exact mechanism by which inhibin affects activin-dependent responses is not clearly understood. Here, we show that inhibin interferes with the activin-specific part of the signaling cascade for 3TP promoter activation, whereas it does not interfere with TGF β signaling. Identical results were obtained by Gonzalez-Manchon and Vale using growth inhibition of CHO cells as a parameter (11). As the signaling pathways activated by activin and TGF β for both of these responses are nearly identical (42), this suggests that the activin-receptor complex is the primary target for inhibin action. We have confirmed the latter hypothesis by showing that inhibin is unable to suppress 3TP promoter activity induced by a constitutively active activin type IB receptor. In addition, we demonstrated that inhibin can displace labeled activin from the ActRII in activin-receptor-transfected COS cells and in wild-type CHO cells. The potency of inhibin is 6–30% that of activin depending on the type II receptor present. This is in accordance with the smaller effect of inhibin, compared to activin, on 3TP-Lux reporter and *junB* gene expression in these cells. Thus, the biological effects of inhibin in CHO cells can be explained on the basis of displacement of activin from the ActRII, as postulated by Xu *et al.* (13). This argues against the existence of a separate inhibin receptor pathway in CHO cells. However, this does not completely exclude the possibility that inhibin, via a separate inhibin receptor pathway, interferes with activin signaling at the level of the activin-receptor complex, *e.g.* via homologs of TRIP-1 that specifically interact with type II receptors (46).

Our results indicate that inhibin cannot interact with ActRI directly or after it has formed a complex with the ActRII. This in contrast to activin, which can interact with the ActRI after it is complexed to a ActRII. Two observations support this conclusion. Firstly, if inhibin interacts with the ActRI directly, it would displace labeled activin from the ActRI and not from ActRII. This is clearly not the case, because the ratio of labeled activin cross-linked to ActRI compared to that

cross-linked to ActRII does not change (in either CHO or COS-1 cells) in the presence of unlabeled inhibin compared to the ratio in the absence of competitor. Thus, displacement of labeled activin from ActRI by inhibin is indirect and is due to displacement of labeled activin from ActRII, which is in line with previous observations (13, 21, 24, 25). Secondly, we observed that unlabeled inhibin behaves differently from unlabeled activin in COS-1 cells overexpressing ActRs. This phenomenon can only be explained if inhibin that is bound to ActRIIs is unable to interact with ActRI and if an excess of ActRIIs over ActRIs is present in transfected COS-1 cells. In that case, unlabeled activin binds to both receptors in a ternary complex, leaving predominantly unoccupied type II receptors for the binding of labeled activin. In this way activin affects the ratio of labeled activin cross-linked to ActRI and ActRII. In the presence of unlabeled inhibin, however, no ternary complex is formed between inhibin complexed to ActRII and the type I receptors. Thus, the type I receptors are not occupied, making it possible that labeled activin can bind equally well to type I and type II receptors, as is the case in the absence of inhibin. CHO cells may have no excess of type II receptors, causing a similar suppression of labeled activin bound to both types of activin receptors by activin and inhibin.

The fact that inhibin cannot interact with the ActRI is in line with studies performed by Xu and co-workers (13). They showed that inhibin only interacts via its β -subunit with ActRII and that the inhibin α -subunit cannot interact with any ActR. This suggests that the α -subunit of inhibin has lost domains that are important for activin receptor interaction and that the α -subunit only prevents receptor dimerization. This is further supported by the fact that cleavage of the inhibin α -subunit to its mature form is not required for inhibin action, whereas cleavage of the inhibin β -subunit is a prerequisite for activin action (47). In addition, compared to the β -subunit gene, the α -subunit displays much greater genetic variability between species (Table 1). All of these features indicate the separate position of inhibin in the TGF β superfamily and support its unique mechanism of action.

The displacement studies further show that ActRIIB is a better target for competition with inhibin than is ActRIIA. This is in line with previous observations by Mathews *et al.* (21) and Attisano *et al.* (24). However, in contrast to results observed by Attisano (24), our data indicate that inhibin is almost as potent as activin in displacing labeled activin from

ActRIIB₂. This may be due to the presence of a type I receptor in our experiments. Recently, Xu and co-workers reported that inhibin was almost as effective as activin in displacing labeled activin from ActRIIA (13). We do not have an explanation for this discrepancy with our present data; the results of competition experiments may depend on the conditions and cell type used and on the expression levels of the ActRs. Unfortunately, Xu *et al.* (13) did not include ActRIIB in their study, so a direct comparison with our data cannot be made. In general, however, the data reported by us and others show that inhibin can displace activin from ActRIIs (13, 21, 24, 25), but not from ActRIs (13).

This competition model allows for a complex and intricate regulation of target cell activities, depending on the relative production of α - and β -subunits and the relative expression of ActRIIA and ActRIIB_{2/4}. Unfortunately, we were unable to determine the ActRIIA/ActRIIB mRNA ratio for CHO cells due to the low level of expression of both ActRIIs. However, the human erythroid cell line K562, which is highly sensitive to inhibin (5), shows a high ActRIIB₂/ActRIIA ratio (van Schaik, R. H. N., unpublished results), which is in line with the idea that the ActRIIB is the ActRII that is most sensitive to inhibin.

The current competition model can explain most data on inhibin action. However, it is difficult to envisage how both inhibin and activin can inhibit a response in the same cell (48, 49) and how inhibin can stimulate a response in the presumed absence of endogenous activin (50). Further, complete insensitivity to inhibin in cells that are responsive to activin (11, 14, 15) may be explained if these cells express the inhibin-insensitive ActRIIA or, alternatively, if they express high levels of ActRIIs so that physiological concentrations of inhibin cannot displace activin. However, it may be necessary to postulate a separate inhibin receptor pathway to explain all of these observations.

The similarity of the intracellular domains of ActRIB and TGF β RI suggests that the activation of these receptors by type II receptors and the signaling pathway downstream of these receptors are identical (42). Indeed, analogous to the TGF β RI (44), the introduction of an aspartic acid for threonine 206 in the GS box of the ActRIB leads to constitutive activation of the kinase domain of this receptor (18)). This supports the view that modifications in or near the GS box are a general mechanism for activation of type I receptors of the TGF β superfamily. Under normal conditions this occurs through phosphorylation of the GS box by the type II receptor (17, 19). It is noteworthy that the introduction of aspartate in the ActRIB results in complete activation of the downstream pathway, because addition of activin to CHO cells transfected with this mutant receptor does not lead to a further increase in the 3TP luciferase response. A similar mutation in TGF β RI resulted in only partial activation of the downstream pathway (44). This suggests that the mechanism or the threshold for activation of the type I receptor by the type II receptor for activin is slightly different from that for TGF β .

In conclusion, we showed that inhibin blocks activin signaling in CHO cells at the level of the activin-receptor complex, most likely through competition with activin at the

TABLE 1. Conservation of inhibin subunit aminoacid sequences among different species

	Inhibin α	Inhibin β_A	Inhibin β_B
Human	100	100	100
Porcine	84	100	99
Bovine	86	100	100
Rat	80	100	97
Mouse	78	100	97

Only the mature peptides were considered. For each inhibin subunit the identity of the peptides is presented as a percentage of the human sequence. The sequences used were obtained from the SWISSPROT database (accession no. P05111, P08476, P09529; P04087, P03970, P04088; P07994, P07995, P42917; P17490, P18331, P17491; and Q04997, Q04998, Q04999; for human, porcine, bovine, rat and mouse inhibin α , β_A , and β_B proteins, respectively).

ActRIIs; the ActRIIBs are more sensitive. Whether inhibin acts via a similar mechanism in other cell types or via a separate inhibin receptor pathway remains to be determined. In this regard it will be of particular interest to investigate gonadotropic cells, because this cell type is very sensitive to inhibin.

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Note Added in Proof

After submission of the manuscript, Lebrun and Vale showed evidence confirming our hypothesis that overexpression of ActRIIs in K562 cells results in loss of sensitivity to inhibin (Lebrun, J. J., and W. W. Vale, Activin and inhibin have antagonistic effects on ligand dependent heterodimerization of the type I and type II activin receptors on human erythroid differentiation, *Mol Cell Biol*, 17:1682–1691).

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