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Activin and Inhibin Have Antagonistic Effects on Ligand-Dependent Heteromerization of the Type I and Type II Activin Receptors and Human Erythroid Differentiation

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Activins and inhibins belong to the transforming growth factor β (TGF-β)-like superfamily and exert their effects on a broad range of cellular targets by modulating cell differentiation and proliferation. Members of this family interact with two structurally related classes of receptors (type I and type II), both containing a serine/threonine kinase domain. When expressed alone, the type II but not the type I activin receptor can bind activin. However, the presence of a type I receptor is required for signaling. For TGF-β1, ligand binding to the type II receptor results in the recruitment and transphosphorylation of the type I receptor. Transient overexpression of the two types of activin receptor results in ligand-independent receptor heteromerization and activation. Nevertheless, activin addition to the transfected cells increased complex formation between the two receptors, suggesting a mechanism of action similar to that observed for the TGF-β receptor. In the present study, we generated a stable cell line, overexpressing the two types of activin receptor upon induction, in the human erythroleukemia cell line K562. We demonstrate here that activin specifically induces heteromer formation between the type I and type II receptors in a time-dependent manner. Using this stable line, we analyzed the effects of activin and inhibin on human erythroid differentiation. Our results indicate that activin signal transduction mediated through its type I and type II receptors results in an increase in the hemoglobin content of the cells and limits their proliferation. Finally, using cell lines that can be induced to overexpress ActRII and ActRIB or ActRIB only, we show that the inhibin antagonistic effects on activin-induced biological responses are mediated through a competition for the type II activin receptor but also require the presence of an inhibin-specific binding component.

Activins, inhibins, transforming growth factor β (TGF-β), Mullerian inhibiting hormone, multiple bone morphogenetic proteins, the *Drosophila* decapentapegic and 60A gene products, and *Xenopus* Vg1 belong to a large superfamily of growth factors. Activins and inhibins are structurally related dimeric molecules which share common β subunits and exert mutually antagonistic effects on their target tissues (20, 23, 28). Activins modulate cell differentiation, cell proliferation, and specific functions of a broad range of cell types (4, 10, 16). In the human erythroleukemia cell line K562, activin can mimic the early stages of erythropoiesis by inducing hemoglobin synthesis (41) at the level of mRNA and protein (26). In K562 cells, inhibin can antagonize the effect of activin on hemoglobin synthesis in a dose-dependent manner (41). Activin also promotes the proliferation of purified erythroid progenitor cells both in vitro (5, 21, 41) and in vivo (24, 27), an effect which is mediated indirectly through both monocytes and T lymphocytes (26). In K562 cells, DNA synthesis is transiently arrested after activin treatment for 24 h, but the cells recover after 3 days, and an increase in DNA synthesis is observed after 5 days (25).

Activins interact with two structurally related receptors (type I and type II), both of which contain an extracellular ligand binding domain, a single transmembrane domain, and an intracellular serine/threonine kinase domain (1, 17, 29). Two type II activin receptors have been identified, ActRII (17) and ActRIIB (1, 18), as well as two putative type I activin receptors (ActRI or ALK2 and ActRIB or ALK4) (1, 31). Binding experiments in COS cells indicate that both ActRII and ActRIIB exhibit high affinity for activin (1, 17, 18, 31). The type I and IB receptors cannot bind activin when expressed alone and require type II receptor coexpression for ligand binding (2, 11, 31). The presence of a type I receptor, however, is required for signaling, and this suggests that the functional form of the activin receptor is a complex between activin and both types of activin receptors (6). Cross-linking experiments using radioiodinated activin verified that the two types of receptors can form a stable complex with activin (2, 19, 30).

Recent studies indicated that TGF-β binding to a constitutively phosphorylated type II receptor results in the recruitment of the type I receptor into the complex and subsequent phosphorylation of the type I receptor in a type II-dependent manner (7, 8, 34, 37). In mink lung cells transiently transfected with the cDNAs encoding both a type I and a type II activin receptor (ActRIB and ActRIIB), activin stimulation results in an increase in receptor association (3) and, therefore, supports a mechanism of action similar to that of the TGF-β receptor. Nevertheless, this study also revealed a strong ligand-independent interaction between the two receptors in unstimulated cells (3). To address this issue as well as to investigate the molecular mechanism of activin action on erythroid differentiation, we generated a system in K562 cells in which overexpression of a type I (ActRIB) and a type II (ActRII) activin receptor is controlled by an inducible promoter upon activation by isopropyl-β-D-thiogalactopyranoside (IPTG). With this inducible cell line we can now demonstrate that in the absence of activin, no detectable association is observed between the type I and type II activin receptors, despite their overexpression. Upon activin stimulation, a heteromer between ActRIB

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and ActRII is rapidly formed. We also show that increased expression of the activin receptors in these cells leads to a ligand-dependent effect on several activin biological responses, including activation of an activin-responsive reporter construct (3TPLux), increased cellular hemoglobin content of the cells, and inhibition of cell proliferation. Finally, we show that inhibin can bind ActRII and oppose these activin-induced effects both in ActRIB-overexpressing and in normal K562 cells but not in cells overexpressing both the type IB and type II receptors, even at high concentrations of inhibin. This indicates that activin and inhibin compete for ActRII and that this competition is required but not sufficient to explain the antagonistic effect of inhibin on activin-induced erythroid differentiation and inhibition of cell proliferation. Indeed, cross-linking experiments in both normal K562 and KAR6 cells revealed the presence of a specific inhibin binding subunit, which represents the first evidence of the existence of a selective inhibin receptor component.

MATERIALS AND METHODS

Materials. Recombinant activin A and inhibin A were generously supplied by J. Mather (Genentech, Inc.). Rabbit anti-ActRIB (α-ActRIB) serum (277-143C) was raised against the carboxy-terminal end of ActRIB (amino acids 493 to 505), and rabbit α-ActRII serum (238-199D) was raised against the carboxy-terminal end of ActRII (amino acids 482 to 494). Both antisera were affinity purified prior to use.

Establishment of the stable cell lines KAR6 and KAR13. ActRIB and ActRII were subcloned into the *lac* operator containing vector PRSVI-CAT (Lac-Switch Inducible Mammalian Expression System, catalog no. 217450; Stratagene). For KAR6 cells, both PRSVI-ActRIB and PRSVI-ActRII were cotransfected into K562 cells by electroporation (Bio-Rad Gene Pulser, 960 μF, 0.22 kV) with the *lac* repressor containing vector p3′SS (Stratagene, catalog no. 217450), while for KAR13 cells, only PRSVI-ActRIB was cotransfected with p3′SS. Following transfection, cells were resuspended in 10 ml of complete medium (RPMI medium containing 10% fetal calf serum). Two days after transfection, G418 (1,500 μg/ml) and hygromycin (300 μg/ml) were added to the culture medium. After 3 weeks of selection, positive clones were purified by dilution cloning and analyzed by Western blotting for their ability to express the activin receptors following 12 h of stimulation by the inducer IPTG (Sigma, catalog no. I-6758).

3TPLux transcriptional assay. All cell lines were grown in complete medium. For transfections, 2×10^7 cells were resuspended in 500 μl of HEPES dissociation buffer (HDB) before electroporation (Bio-Rad Gene Pulser, 960 μF, 0.22 kV) with 20 μg of 3TPLux plasmid (kindly provided by Joan Massagué) and 10 μg of CMV-β-galactosidase plasmid. The cells were then resuspended in 20 ml of complete medium and incubated at 5% CO_2, 37°C overnight. The following day, cells were plated at 10^6 cells/ml in starvation medium (RPMI medium with no fetal calf serum) in the presence or absence of IPTG (5 mM) and were treated or not with activin or inhibin at the indicated concentrations for 24 h before being harvested. Then, cells were lysed in 100 μl of lysis buffer (1% Triton X-100, 15 mM $MgSO_4$, 4 mM EGTA, 1 mM dithiothreitol, 25 mM glycylglycine) for 30 min on ice, and the luciferase activity of each lysate was measured and normalized to the relative β-galactosidase activity.

Immunoprecipitations. Cells were stimulated with activin or inhibin at the indicated concentrations and times. The cells were then lysed in 1 ml of lysis buffer (50 mM Tris-HCl [pH 7.5], 5 mM EDTA, 150 mM NaCl, 10% glycerol, 0.5% Nonidet P-40) containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 μg of pepstatin A/ml, 2 μg of leupeptin/ml, 5 μg of aprotinin/ml) for 10 min at 4°C. The insoluble material was discarded following centrifugation at 12,000 × *g* for 15 min. Cell lysates were immunoprecipitated overnight at 4°C with an α-ActRIB (277-143C) or an α-ActRII (238-199D) antibody and 30 μl of protein A-Sepharose beads (10% in lysis buffer). Samples were then washed three times in 1 ml of washing buffer (50 mM Tris-HCl [pH 7.5], 2 mM EDTA, 150 mM NaCl, 10% glycerol) and eluted in 20 μl of sodium dodecyl sulfate (SDS) loading buffer (20% glycerol, 10% β-mercaptoethanol [βME], 4.6% SDS, 0.125 M Tris-HCl [pH 6.8]).

Western blot analysis. Proteins were separated on a 7.5% polyacrylamide gel, transferred onto nitrocellulose, and incubated with either an α-ActRIB or α-ActRII antibody (at 1/1,000 dilution) overnight at 4°C. Following incubation, the membranes were washed twice for 15 min in washing buffer (50 mM Tris-HCl [pH 7.6], 200 mM NaCl, 0.05% Tween 20) and incubated with a secondary anti-rabbit antibody coupled to peroxidase (α-rabbit immunoglobulin horseradish peroxidase [NA934, Amersham] at a 1/4,000 dilution) for 1 h at room temperature. Then, the membranes were washed four times for 30 min in washing buffer, and immunoreactivity was normalized by chemiluminescence (ECL kit, catalog no. RPN 2106; Amersham) according to the manufacturer's instructions.

Membrane stripping. Following the first round of immunodetection, membranes were stripped for 15 min at 55°C in stripping buffer (100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl [pH 6.7]) and were washed for several hours in washing buffer before used again for immunodetection.

Binding and Scatchard analysis. Cells (K562, KAR6, and KAR13) were incubated overnight in RPMI medium with or without 5 mM IPTG (KAR6 and KAR13 cells only). After three washes with HDB, the cells were resuspended in 0.4 ml of binding buffer (HDB, 0.1% bovine serum albumin, 5 mM $MgSO_4$, 1.2 mM $CaCl_2$) at 2×10^6 cells/ml and incubated with 0.5 nM ^{125}I-activin (60 μCi/μg) and increased concentrations of cold activin (0, 0.06, 0.6, 6.2, 62.5, 625 ng/ml) for 90 min at room temperature. Cells were then washed twice with 1 ml of binding buffer and centrifuged at 12,000 × *g* for 5 min, and the cell pellets were counted in a gamma counter. Scatchard analysis and estimation of the number of receptors per cell was determined using the LIGAND program.

Cross-linking experiments. A total of 4×10^6 cells (K562, KAR6, and KAR13) were stimulated overnight with 5 mM IPTG in RPMI medium. Cells were then washed three times in HDB, resuspended in 0.5 ml of cross-linking buffer (HDB, 0.1% bovine serum albumin, 5 mM $MgSO_4$, 1.2 mM $CaCl_2$) containing 1 nM ^{125}I-activin or ^{125}I-inhibin (60 μCi/μg) and incubated in the presence or absence of a 60-fold molar excess of cold activin or inhibin for 80 min at room temperature. Cells were then washed with 1 ml of HDB and incubated in 0.5 ml of HDB with 500 μM disuccinimidyl suberate for 30 min on ice. The reaction was quenched with 1 ml of 50 mM Tris–100 mM NaCl, pH 7.5, and the cells were centrifuged at 12,000 × *g* for 5 min. Cell pellets were solubilized in 100 μl of 50 mM Tris–1% Triton X-100, pH 7.5, and incubated on ice for 45 min. Following centrifugation (12,000 × *g*, 5 min), the insoluble material was discarded, and the supernatant was diluted in 400 μl of lysis buffer and immunoprecipitated with the α-ActRII antibody as described above. Samples were separated by SDS–7.5% polyacrylamide gel electrophoresis (PAGE) and the gels dried before being subjected to autoradiography.

Benzidine staining. Cells were incubated at a concentration of 1.5×10^5/ml in complete medium in the presence or absence of the inducer IPTG (5 mM) and stimulated with activin (1 nM), inhibin (1 nM), or both activin and inhibin (1/4 molar ratio). Four days after stimulation 40 μl of the cell culture was added to 10 μl of fresh benzidine solution (1.5 mg of benzidine [Sigma, catalog no. B-3503] in 1 ml of H_2O, 25 μl of 30% H_2O_2, and 50 μl of glacial acetic acid) for 5 min, and the number of blue-stained cells was counted by using light microscopy.

Proliferation assays. Cells were plated in triplicates in complete medium, at a concentration of 10^4 cells/ml in 96-well plates, incubated in the presence or absence of 5 mM IPTG, and stimulated with 1 nM activin A or inhibin A or 1 nM activin plus 4 nM inhibin for 1 to 6 days. Cell proliferation was assessed by using the nonradioactive MTT cell proliferation assay for eukaryotic cells (Cell Titer 96; Promega catalog no. G 4000).

RESULTS

IPTG-induced expression of the activin receptors (ActRIB and ActRII). K562 cells express low but detectable levels of endogenous ActRII and ActRIIB immunoreactive receptor protein. However, we failed to detect any immunoreactive ActRI or ActRIB, despite a normal expression of their respective mRNAs (31). Cross-linking experiments using radioiodinated activin in K562 cells revealed that both ActRIB and ActRI are present in a complex with the type II receptor (32). In K562 cells, transfection of the kinase-deficient ActRIB but not the kinase-deficient ActRI can block activin induction of 3TPLux activity (32), suggesting that this effect of activin is mediated through ActRIB rather than ActRI. Because of their low levels of expression, the study of the interactions between the two types of activin receptors is a difficult process. Our first attempts to generate a stable cell line overexpressing ActRIB and ActRII were unsuccessful, possibly due to a ligand-independent interaction of the two receptors leading to inhibition of cell proliferation. Therefore, we generated an inducible K562 cell line which could overexpress both ActRIB and ActRII (clone KAR6) upon activation by an inducer (IPTG). The cDNAs encoding ActRIB and ActRII were subcloned into a *lac* operator containing vector pORSVICAT and cotransfected with a *lac* repressor containing vector P3′SS. Under normal conditions, the stable clones express only endogenous levels of ActRIB or ActRII due to the inhibition of the *lac* operator by the *lac* repressor. A few hours before analysis, overexpression of both receptors is rapidly turned on by addition of IPTG, a synthetic inducer, which binds to the *lac* repressor and therefore alters its conformation resulting in an inability to bind to the *lac* operator sequence.

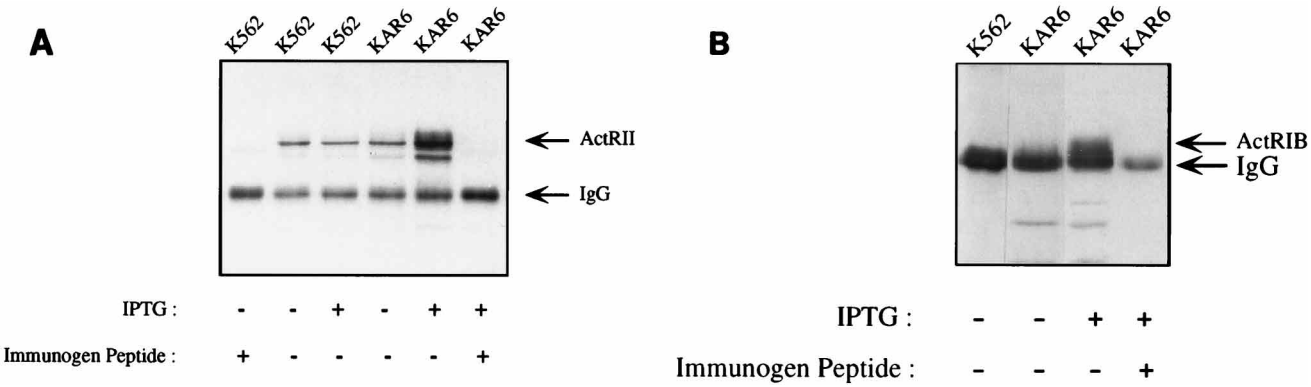

FIG. 1. IPTG-induced overexpression of ActRIB and ActRII in normal K562 cells and in the stable cell line KAR6. Cells were starved overnight in the absence (−) or presence (+) of 5 mM IPTG and lysed before being immunoprecipitated with an antibody against ActRII (A) or ActRIB (B) in the presence or absence of the corresponding immunogen peptide. The immunoprecipitated proteins were separated by SDS-PAGE, transferred to a nitrocellulose membrane, and analyzed by Western blotting with an antibody against ActRII (panel A) or ActRIB (panel B). The positions of ActRIB (60 kDa), ActRII (80 kDa), and the heavy chains of the IgG (55 kDa) are indicated by arrows on the right side.

As shown in Fig. 1A, in the absence of IPTG, the level of expression of ActRII in the stable cell line KAR6 is slightly higher than that observed in normal K562 cells (lanes 2, 3, and 4). Upon addition of IPTG, the level of expression of ActRII is clearly increased (lane 5). Interestingly, the signal corresponding to ActRII appears as a doublet (lane 5). The second band of lower molecular weight can also be detected in normal K562 cells or noninduced KAR6 cells, following longer exposure of the film (data not shown) and may correspond to a posttranslational modification of ActRII. The specificity of the signal was demonstrated by coincubating the cell lysates with the immunogen peptide during the immunoprecipitation step (lanes 1 and 6). The additional band (55 kDa) observed in all lanes below the ActRII signal corresponds to the nonspecific signal generated by the heavy chains of the immunoglobulin G (IgG) used during the immunoprecipitation step. In the absence of IPTG, no detectable expression of ActRIB is seen in normal K562 or KAR6 cells (Fig. 1B, lanes 1 and 2). After induction by IPTG, expression of ActRIB is induced in the stable cell line (Fig. 1B, lane 3). The specificity of the observed signal was verified by coincubation of the immunoprecipitates with the immunogen peptide (Fig. 1B, lane 4). As shown in Table 1, Scatchard analysis revealed that normal K562 cells express about 550 receptors per cell. In noninduced KAR6 cells, this number is slightly higher (740 receptors per cell) and is probably due to incomplete repression of exogenous ActRII expression. By contrast, in IPTG-induced KAR6 cells the receptor number per cell is 3.5-fold greater (1,882 receptors per cell) than in normal K562 cells, confirming the higher level of ActRII expression observed in Fig. 1A. Together, these results indicate that IPTG induction results in increased expression of both ActRIB and ActRII as determined by both immunodetection and Scatchard analysis. This system provides a useful tool to analyze the interactions between the two types of activin receptors as well as the activin effect on erythroid differentiation mediated through these receptors.

TABLE 1. Numbers of receptors expressed in K562, KAR6, and KAR13 cells in the presence or absence of the inducer IPTG[a]

Cell line	No. of receptors/cell	Range[b]
K562	542	342–855
KAR6		
Without IPTG	740	370–1,450
With IPTG	1,882	1,600–2,196
KAR13		
Without IPTG	827	542–1,255
With IPTG	941	456–1,881

[a] For the Scatchard analysis, cells were incubated with ^{125}I-activin in the presence of increasing doses of cold activin. The estimation of the number of receptor per cell was determined by using the LIGAND program.
[b] Confidence interval.

Activin-induced association of the type I and II activin receptors. As opposed to the type II receptor, the type I activin receptor requires coexpression of the type II receptor for ligand binding in COS cells (1, 17, 18, 31). Indeed, functional activin receptor complexes are heteromers containing both the type I and II activin receptors and activin (6, 31). Recently, studies of activin receptors (ActRIB and ActRIIB) transiently transfected in mink lung cells indicated a strong ligand-independent interaction between the two receptors (3), further supporting the possibility of a constitutive association between ActRIB and ActRIIB. To investigate this, the level of type I and II activin receptor association was measured in IPTG-induced KAR6 cells, in the presence or absence of 1 nM activin for 15 min, and receptor heteromerization was analyzed by immunoprecipitation and Western blotting. Cell lysates were immunoprecipitated with an antibody directed against ActRIB and immunoblotted with an antibody against ActRII (Fig. 2A, upper panel). As shown in lane 1, in the absence of ligand no detectable complex between the two receptors is observed, but when cells are stimulated with activin, a complex between the two receptors is formed (lane 2). Similar levels of ActRIB were observed in control and activin-treated cells, as shown in the lower panel of Fig. 2A where the same membrane has been stripped and reprobed with an antibody against ActRIB. The specificity of the observed signal was ensured by coincubating the immunoprecipitation reaction of stimulated cells with the ActRIB immunogen peptide (Fig. 2A, lane 3). A similar result (activin-dependent heteromerization) was observed when cell lysates were immunoprecipitated with an anti-ActRII antibody and immunoblotted with an anti-ActRIB antibody (Fig. 2B). These results clearly demonstrate that ligand stimulation rapidly leads to heteromeric association of type I and II activin receptors, with no detectable association between the two receptors in the absence of ligand.

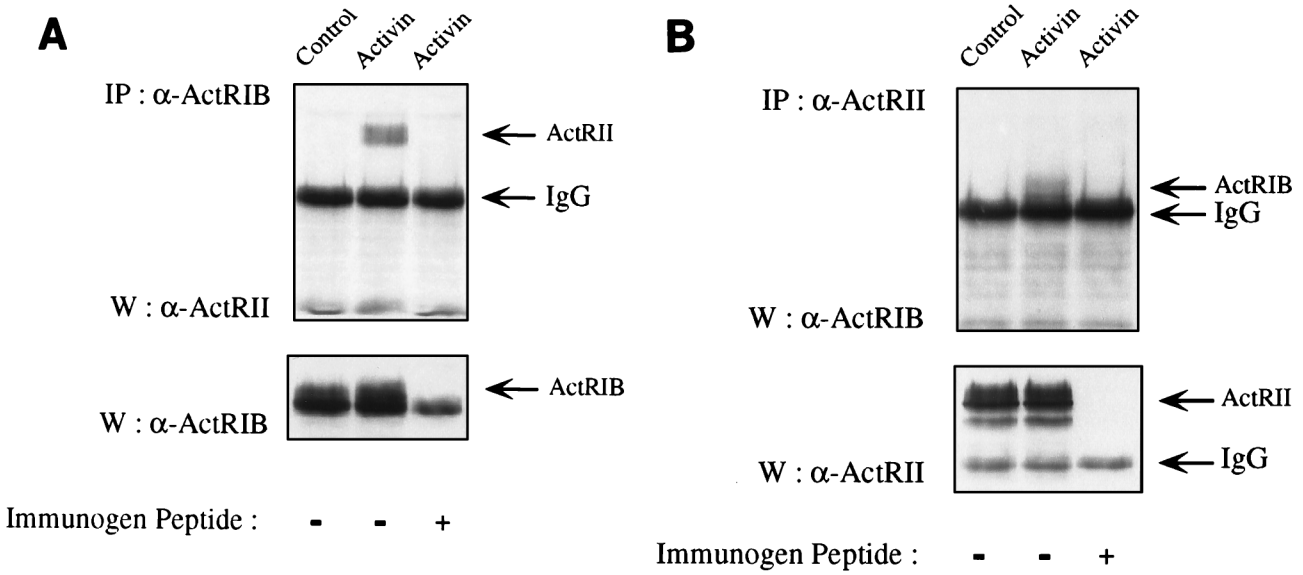

FIG. 2. Ligand-dependent association of ActRIB and ActRII. IPTG-induced KAR6 cells were stimulated with 0 or 1 nM activin A for 15 min. Cell lysates were immunoprecipitated with an anti-ActRIB (A) antibody or an anti-ActRII (B) antibody in the presence (+) or absence (−) of the corresponding immunogen peptide. Immunoprecipitated proteins were separated by SDS-PAGE and analyzed by Western blotting by using an anti-ActRII antibody (panel A, upper) or anti-ActRIB antibody (panel B, upper). The membranes were then stripped and reprobed with an anti-ActRII (panel B, lower) or anti-ActRIB (panel A, lower). The positions of the two types of activin receptor and the heavy chains of IgG are indicated to the right side by arrows.

Kinetics of receptor heteromerization. To determine the time course of association between the two receptors, we treated IPTG-induced KAR6 cells with 1 nM activin for 0 to 90 min. As shown in Fig. 3 (upper panel), no association is detected in the absence of ligand. However, within 1 min of activin stimulation, a complex is observed between ActRII and ActRIB. The association between the two receptors increases to reach its maximum after 15 min of activin stimulation and then decreases after 30 min. In the lower panel, the membranes were reprobed with an anti-ActRIB antibody to demonstrate equivalent immunoprecipitation of ActRIB at each time point. This indicates that activin receptor heteromerization is a very rapid and transient event occurring a few minutes after ligand stimulation.

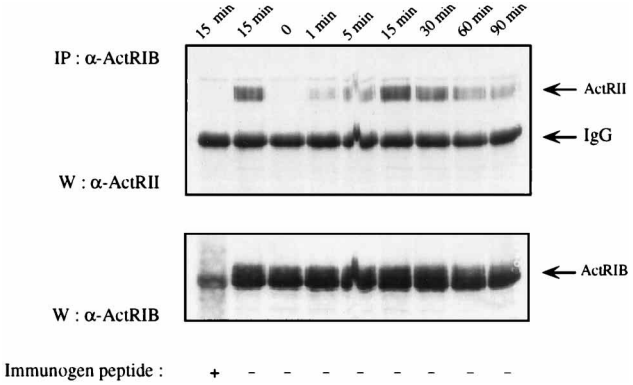

FIG. 3. Time course of the activin-dependent formation of a heterodimer between ActRIB and ActRII. IPTG-induced KAR6 cells were stimulated with 1 nM activin for the indicated times. Cell lysates were immunoprecipitated with an anti-ActRIB antibody, separated by SDS-PAGE, and immunoblotted with an anti-ActRII (upper panel) or anti-ActRIB (lower panel) antiserum. The positions of the type I and type II activin receptors and the heavy chains of the IgG are indicated by arrows on the right side. On the first lane, the immunogen peptide was added to the immunoprecipitation reaction as a control.

ActRIB and ActRII overexpression increases activin-induced transcriptional activity. ActRIB has been shown to mediate the activin-induced transcriptional activity of the reporter construct 3TPLux, which contains a region of the plasminogen activator inhibitor promoter fragment as well as three tetradecanoyl phorbol acetate responsive elements coupled to the luciferase gene (6, 32). To determine if overexpression of the two receptors results in an increase in activin-induced transcriptional activity, K562 and KAR6 cells were transiently transfected with 3TPLux and then stimulated with 1 nM activin in the absence or presence of IPTG. In the absence of IPTG, the activin-induced luciferase activity in KAR6 cells is slightly increased compared to that observed in K562 cells stimulated with activin (Fig. 4), and this result is supported by the data reported in Fig. 1A and Table 1 where the level of ActRII expression is increased even in the absence of IPTG. When KAR6 cells are induced by IPTG, a clear and significant increase in luciferase activity above that of normal K562 or uninduced KAR6 cells is observed in response to activin stimulation. Unlike transient-transfection systems, no constitutive activation of 3TPLux results from the stable overexpression of ActRIB and ActRII in the absence of ligand, thereby demonstrating the strict ligand dependence of our system.

Activin receptor overexpression potentiates the ligand-induced increase in hemoglobin content of KAR6 cells. A hallmark of the effect of activin on erythrocyte precursors is the induction of erythroid differentiation (41). Activin is a hematopoietic growth factor which can induce the differentiation of erythroid progenitor cells to the erythroid lineage, and this effect is antagonized in a dose-dependent manner by inhibin (41). We therefore assessed the roles of both activin and inhibin on hemoglobin synthesis in our system. KAR6 cells were treated with or without IPTG and stimulated for 4 days with 1 nM activin. Then, the hemoglobin content was measured by benzidine staining. As shown in Fig. 5, activin alone significantly increased the percentage of hemoglobin-containing cells in KAR6 cells in the absence of IPTG in a way similar to its

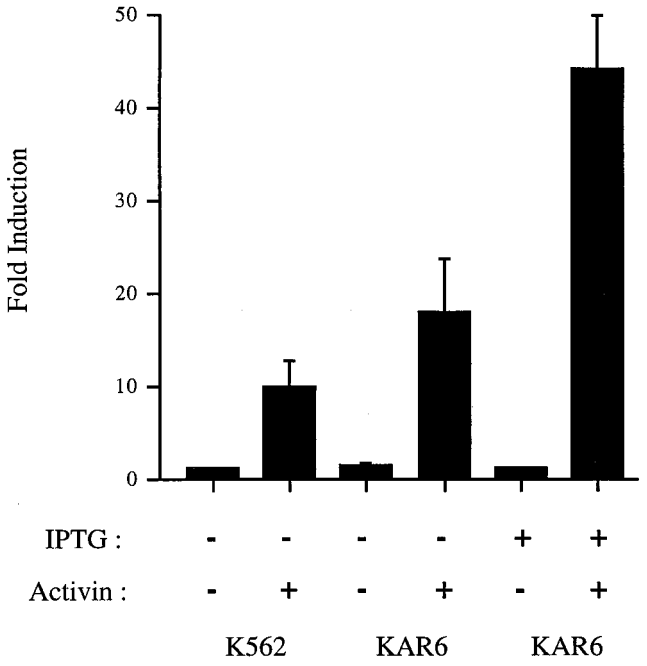

FIG. 4. Ligand-dependent 3TPLux activity. K562 cells and KAR6 cells, induced (+) or uninduced (−) with 5 mM IPTG, were transiently transfected with both the 3TPLux reporter construct and the CMV-β-galactosidase plasmid. Cells were stimulated or not with 1 nM activin A as indicated for 24 h before being lysed. The luciferase activity was normalized to the relative β-galactosidase values and represents means and standard deviations of four independent experiments. Results are expressed as fold induction compared to normal K562 cells cultured in absence of activin.

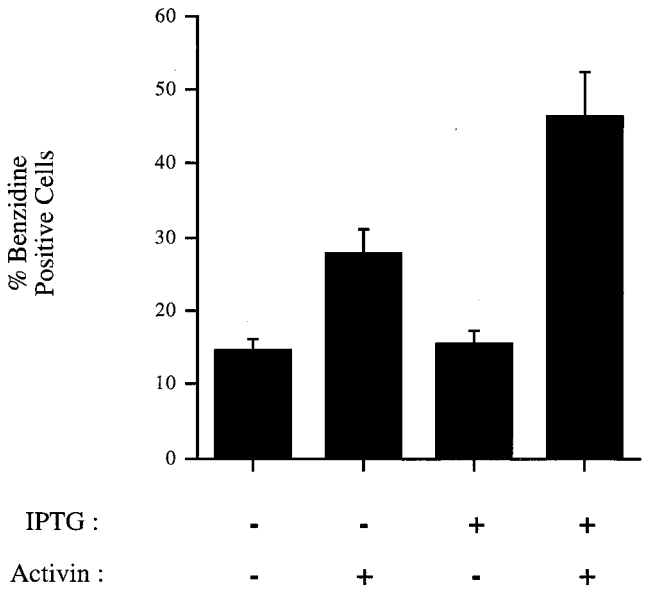

FIG. 5. Activin effect on hemoglobin synthesis. KAR6 cells were induced (+) or uninduced (−) with 5 mM IPTG and stimulated or not with 1 nM activin as indicated for 4 days before being analyzed by benzidine staining. The results are expressed in percentages of blue-stained cells compared to the total cell number. The values represent the means and standard deviations of three separate experiments.

observed effect on normal K562 cells (41). In IPTG-induced KAR6, activin treatment significantly increased the percentage of hemoglobin-containing cells, indicating that the activin effect on erythroid differentiation in K562 cells is directly mediated through its type I and type II receptors. In KAR6 cells, a ligand-independent increase in the number of hemoglobin-containing cells was not observed in the absence of activin, again confirming the specificity of our system.

Effect of inhibin on hemoglobin synthesis in IPTG-induced KAR6 cells overexpressing ActRIB and ActRII. In general, inhibins have been shown to have effects antagonistic to activins in their target tissues. It has been suggested that inhibin, which shares a common β subunit with activin, can antagonize activin effects on hepatocytes by competing for the type II receptor (38). In K562 cells, the stimulatory effect of activin on the number of hemoglobin-containing cells is antagonized by inhibin in a dose-dependent manner (41). In IPTG-induced KAR6 cells, inhibin does not affect either basal or activin-induced increases in the hemoglobin content of the cells at a four times molar excess (Fig. 6). Therefore, overexpression of either ActRIB or ActRII or both prevents the antagonistic effect of inhibin on the activin-stimulated increase in hemoglobin synthesis.

Effect of inhibin on receptor heteromerization. To determine the ability of inhibin to modulate activin-induced complex formation between ActRIB and ActRII, IPTG-induced KAR6 cells were stimulated with 1 nM activin or inhibin or both at different ratios. As shown in Fig. 7 (upper panel), inhibin alone cannot induce complex formation between the two types of activin receptors. Inhibin also does not antagonize activin-induced receptor heteromerization even when present in an eightfold molar excess of activin in this cell line. Subsequent incubation of the membrane with an anti-ActRIB antibody revealed similar amounts of ActRIB in all samples (Fig. 7, lower panel). This indicates that in the presence of high levels of type IB and type II activin receptors, inhibin does not antagonize activin effects on receptor heteromerization and subsequent induction of hemoglobin synthesis (Fig. 6).

Effect of inhibin on activin-induced 3TPLux transcription in KAR6 cells. To examine the effect of inhibin on activin-induced transcriptional activity of 3TPLux, K562 and IPTG-induced KAR6 cells were transiently transfected with the reporter construct (3TPLux). Cells were then stimulated with 250 pM activin, in the presence or absence of increasing concentrations of inhibin. As shown in Fig. 8A and B, inhibin alone does not affect the 3TPLux response in either cell line. However, in normal K562 cells, addition of increasing concentrations of inhibin to the activin-stimulated cells results in a progressive loss of the activin-induced luciferase activity. Indeed, in the presence of an eightfold or more molar excess, inhibin can fully antagonize the activin response on 3TPLux (Fig. 8A). Interestingly, in KAR6 cells inhibin does not exert any antagonistic effect on this 3TPLux activity even at a 128-fold molar excess of inhibin (Fig. 8B). This indicates that overexpression of both the type I and type II activin receptors results in the loss of antagonism by inhibin for activin-induced 3TPLux activity. The lack of effect of high concentrations of inhibin in activin-treated KAR6 cells also indicates that the competition between activin and inhibin for the type II receptor is not sufficient to explain the antagonistic effect of inhibin on activin-induced biological functions. These data suggest the presence and involvement of another molecule, such as an inhibin receptor, in the mediation of this antagonistic effect.

Inhibin competition for the type II activin receptor in cells overexpressing ActRIB. To verify that the loss of antagonistic effects of inhibin is due to the high level of ActRII in KAR6 cells, we generated another inducible cell line (KAR13) in which only ActRIB is overexpressed following IPTG induction.

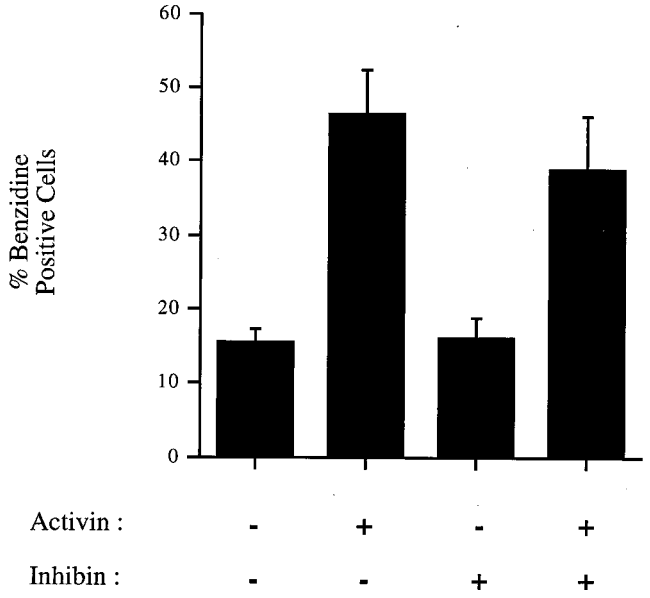

FIG. 6. Effect of inhibin on hemoglobin synthesis. IPTG-induced KAR6 cells were stimulated (or not) with 1 nM activin, 1 nM inhibin, or 1 nM activin plus 4 nM inhibin as indicated for 4 days before being analyzed by benzidine staining. The results are expressed as percentages of blue-stained cells compared to the total cell number. The values represent the means and standard deviations of three separate experiments.

As shown in Fig. 9A, ActRIB expression is induced following treatment with 1 or 5 mM IPTG. The level of ActRII expression remains the same in the presence or absence of IPTG and is not significantly different from that observed in K562 cells (Table 1 and data not shown). As shown in Fig. 9B, activin but not inhibin induces the formation of the heteromeric complex between the two receptors, confirming the previous observations in KAR6 cells. However, in contrast to that seen with

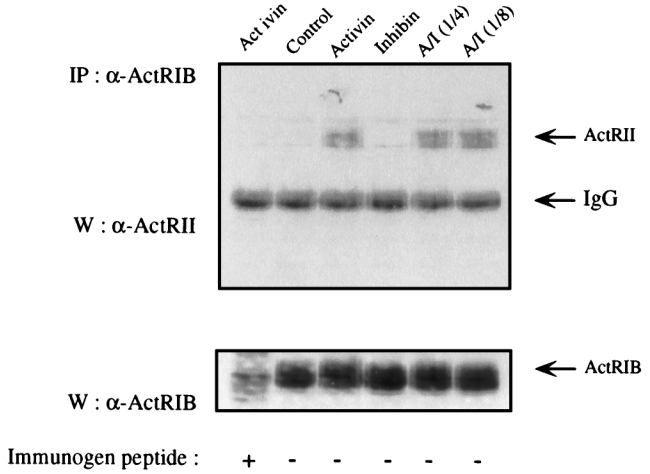

FIG. 7. Effect of inhibin on activin-induced heteromerization between ActRIB and ActRII. IPTG-induced KAR6 cells were stimulated with activin A (1 nM) or inhibin A (1 nM) or both activin A and inhibin A at a 1/4 or a 1/8 molar ratio for 15 min. Cell lysates were immunoprecipitated with an anti-ActRIB antibody, separated by SDS-PAGE, and analyzed by Western blotting with an anti-ActRII (upper panel) or anti-ActRIB (lower panel) antibody. The positions of the type I and type II activin receptors and the heavy chains of IgG are indicated by arrows on the right. On the first lane, the immunogen peptide was added to the immunoprecipitation reaction as a control.

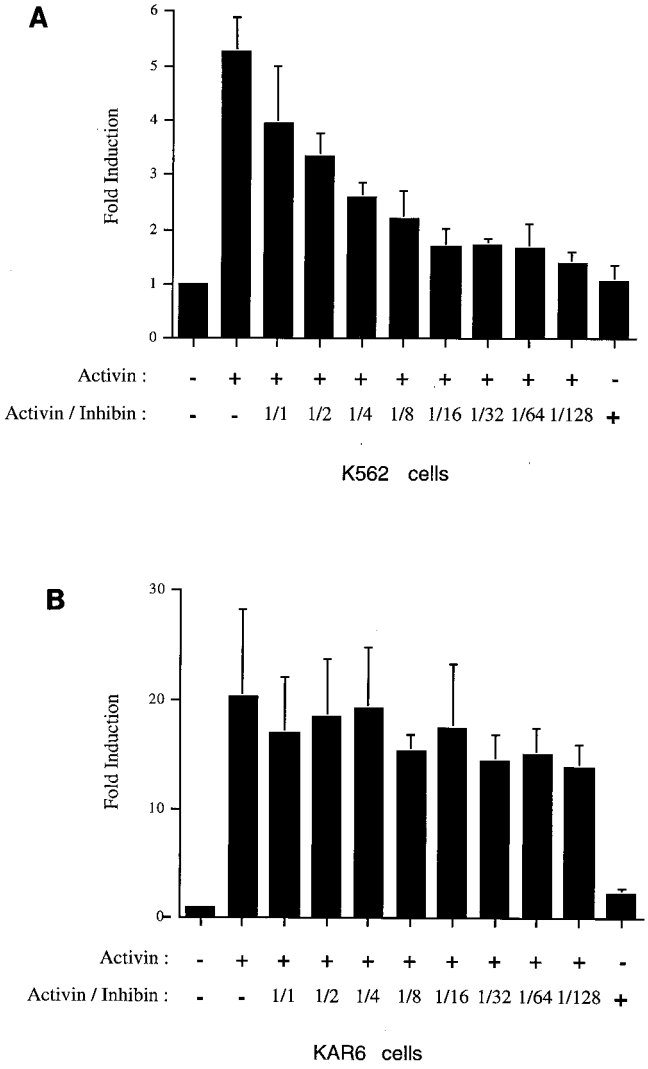

FIG. 8. Antagonist effect of inhibin on activin-induced 3TPLux activity. K562 cells (A) and IPTG-induced KAR6 cells (B) were transiently transfected with the 3TPLux reporter construct and the CMV-β-galactosidase plasmid. Cells were stimulated with 250 pM activin in the presence or absence of the indicated concentrations of inhibin for 24 h. The luciferase activity was normalized to the relative β-galactosidase values and represents means and standard deviations of four independent experiments. Results are expressed as fold induction compared to control cells cultured in the absence of activin.

KAR6 cells, in KAR13 cells, the activin-induced complex formation between ActRIB and ActRII is antagonized by inhibin (Fig. 9B, lane 5). Cross-linking experiments using radioiodinated activin as a tracer in K562, KAR6, and KAR13 cell lines revealed that both cold activin and inhibin can displace the binding of the activin tracer in K562 and KAR13 cells, but only cold activin could exert this effect in KAR6 cells (Fig. 9C), confirming the result observed by Western blot analysis (Fig. 7, and 9B). Finally, we also examined the inhibin effect on 3TPLux transcriptional activity in KAR13 cells. As shown in Fig. 9D, the activin effect on 3TPLux activity is increased in the presence of overexpressed ActRIB (KAR13 cells), probably indicating that in normal K562 cells ActRIB is the limiting factor compared to ActRII, as supported by our Western blot results (Fig. 1A and B). In both K562 and KAR13 cells inhibin does not affect the 3TPLux transcriptional activity by itself, but

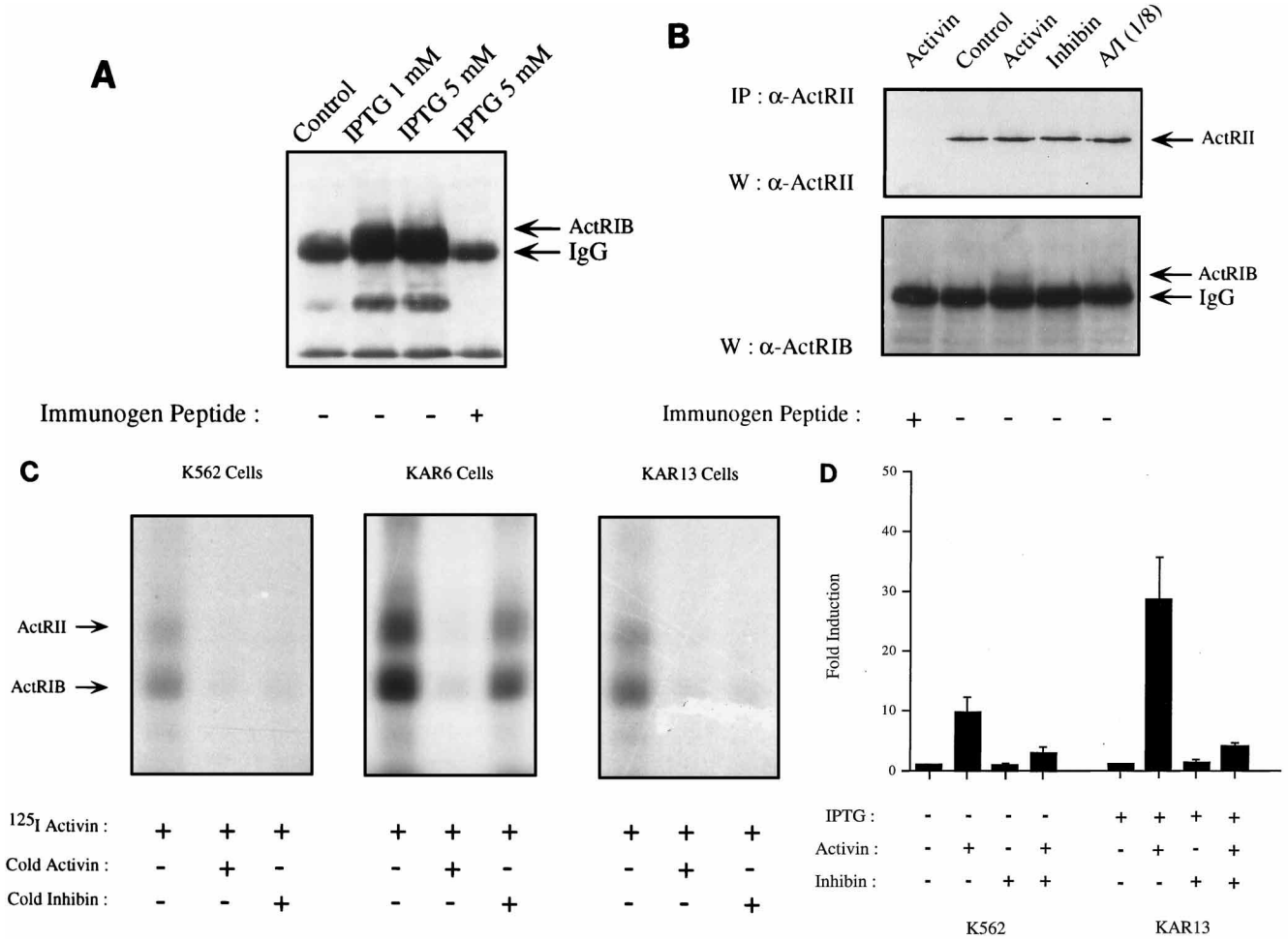

FIG. 9. Antagonist effect of inhibin in KAR13 cells. (A) IPTG-induced overexpression of ActRIB in the stable cell line KAR13. Cells were starved overnight and induced or not with 1 or 5 mM IPTG. Cells were then lysed and immunoprecipitated with an antibody against ActRIB in the presence or absence of the corresponding immunogen peptide. The immunoprecipitated proteins were separated on SDS-PAGE, transferred to a nitrocellulose membrane, and analyzed by Western blotting with an antibody against ActRIB. The positions of ActRIB (60 kDa) and heavy chains of IgG (55 kDa) are indicated by an arrow on the right side. (B) KAR13 cells were stimulated with activin (1 nM) or inhibin (1 nM) or both activin and inhibin at a 1/8 molar ratio for 15 min. Cell lysates were immunoprecipitated with the α-ActRII antibody, separated by SDS-PAGE, analyzed by Western blotting with an α-ActRIB antibody (lower panel), and then stripped and reprobed with an α-ActRII antibody (upper panel). The positions of ActRIB, ActRII, and heavy chains of IgG are indicated by arrows on the right side. On the first lane, the immunogen peptide was added to the immunoprecipitation reaction as a control. (C) K562, KAR6, and KAR13 cells were incubated with ^{125}I-activin in the presence or absence of a 60-fold excess of cold activin or inhibin. Following chemical cross-linking and immunoprecipitation with the α-ActRII antibody, the samples were separated by SDS-PAGE and analyzed by autoradiography. The positions of ActRIB and ActRII are indicated on the left side by arrows. (D) K562 cells and KAR13 cells were transiently transfected with the 3TPLux reporter construct and the CMV-β-galactosidase plasmid. Cells were induced or not with 5 mM IPTG and stimulated with 1 nM activin, 1 nM inhibin, or 1 nM activin plus 8 nM inhibin for 24 h. The luciferase activity was normalized to the relative β-galactosidase values and represents means and standard deviations of four independent experiments. Results are expressed as fold induction compared to normal K562 cells cultured in the absence of activin.

the antagonistic effect of inhibin on activin-induced 3TPLux activity is similar in both cell lines (Fig. 9D) and confirms that the lack of antagonism in KAR6 cells requires overexpression of the type II receptor.

Anti-proliferative effect of activin. In K562 cells, activin has been shown to exert a transient arrest in DNA synthesis after 24 h of treatment, but then the cells recover and the level of [^{3}H]thymidine incorporation returns to normal after 3 days (25). To determine the activin effects on cell proliferation in our IPTG-inducible stable cell line over a several-day period, we used a cell proliferation assay which measures the metabolic activity of the cellular enzymes which convert tetrazolium salt into formazan product, as described previously (22). As shown in Fig. 10A, addition of IPTG to KAR6 cells does not affect cell proliferation. Addition of activin to the cells results in a partial inhibition of cell proliferation, and this effect is enhanced when ActRIB and ActRII expression is induced by IPTG. This verifies the effect of activin as an antiproliferative agent in K562 cells and indicates that this effect can be mediated through the type I and type II receptors. Addition of inhibin does not affect cell proliferation nor does it block the activin-induced inhibition of cell proliferation in KAR6 cells (Fig. 10B). However, in KAR13 cells where only ActRIB is overexpressed, inhibin can fully antagonize activin effects on proliferation at a 1/8 molar ratio (Fig. 10C).

Evidence for the presence of an inhibin receptor in K562 cells. To try to identify the other component required in the mediation of the antagonistic effect of inhibin on activin-induced biological actions, cross-linking experiments using the ^{125}I-activin and inhibin tracers followed by immunoprecipitation with the α-ActRII antibody were conducted in both K562 and KAR6 cells. As shown in Fig. 11, and as previously de-

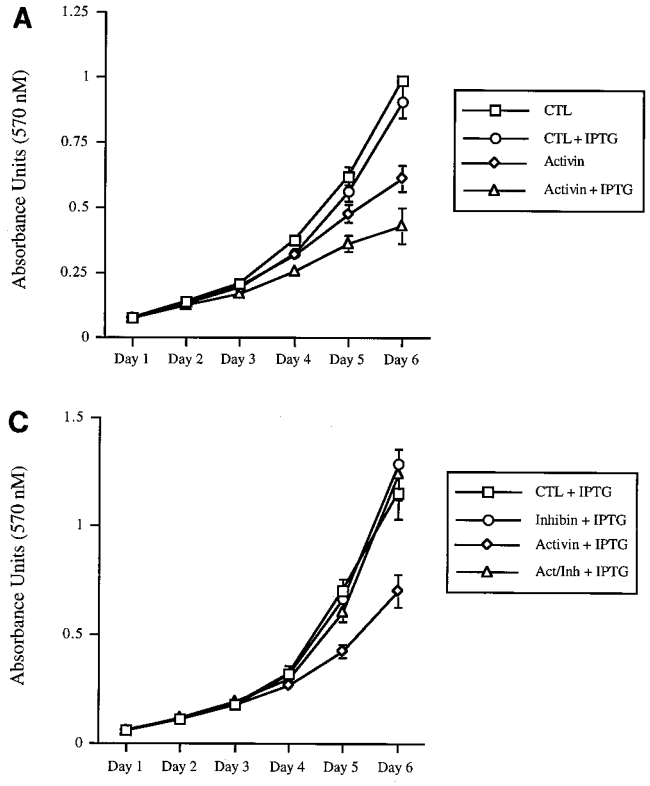

FIG. 10. MTT cell proliferation assay. Cells were incubated in RPMI medium containing 10% fetal calf serum, and cell proliferation was measured by MTT assay in triplicate, according to the manufacturer's instructions. Values represent means and standard deviations of four separate experiments and are expressed in absorbance units. (A) KAR6 cells were cultured in the absence or presence of IPTG and stimulated or not by 1 nM activin A for 1 to 6 days. (B) IPTG-induced KAR6 cells were stimulated with activin (1 nM), inhibin (1 nM), or both activin and inhibin (Act/Inh) (1 and 8 nM, respectively) for 1 to 6 days. (C) IPTG-induced KAR13 cells were stimulated with activin (1 nM), inhibin (1 nM), or both activin and inhibin (1 nM and 8 nM, respectively) for 1 to 6 days.

scribed (Fig. 9C), activin binds to both the type I and type II receptor in both cell lines. Binding of the activin tracer to the two receptors is displaced by an excess of cold activin or cold inhibin. The higher intensity of the signal observed when using KAR6 cells is due to the higher levels of expressed ActRIB and ActRII. The higher-molecular-weight bands of lower intensity probably correspond to nonreduced multimeric forms of these complexes since they are also displaced by an excess of cold ligand. As expected, the inhibin tracer is capable of binding the type II receptor in both cell lines, and binding can be displaced by addition of cold activin or inhibin, but the inhibin tracer fails to bind the activin type I receptor (Fig. 11). However, a higher-molecular-weight protein appears to bind to the inhibin tracer and is also displaced by addition of cold ligand. Due to the

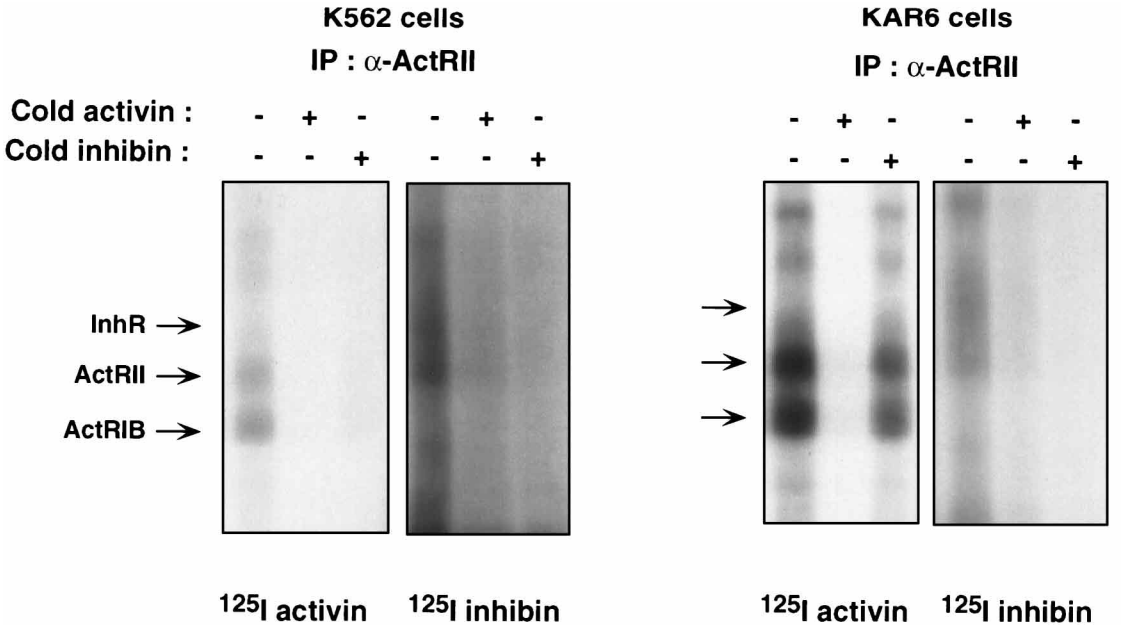

FIG. 11. Evidence for the presence of a inhibin receptor in K562 cells. K562 and IPTG-induced KAR6 cells were incubated with either ^{125}I-activin or ^{125}I-inhibin in the presence or absence of a 60-fold excess of cold activin or inhibin. Following chemical cross-linking and immunoprecipitation with the α-ActRII antibody, the samples were separated by SDS-PAGE and analyzed by autoradiography after 3 weeks of exposure, except for the right panel (^{125}I-inhibin tracer in KAR6 cells) which was exposed for 1 week. The positions of ActRIB and ActRII as well as of the inhibin receptor (InhR) are indicated on the left side by arrows.

higher level of ActRII expression in KAR6 cells, the signal observed for both ActRII and the higher-molecular-weight protein using the inhibin tracer is stronger than in K562 cells (not reflected in Fig. 11 due to the shorter exposure of the KAR6 autoradiogram). These results suggest that following binding to ActRII, inhibin recruits another subunit of higher molecular weight into the complex.

DISCUSSION

The serine/threonine kinase receptors are expressed at very low levels, rendering their study difficult. We report here the generation and the validation of a model system in an erythroleukemia cell line, K562, to investigate activin receptor signaling mechanisms and subsequent effects on erythroid differentiation. Our first attempt to generate a constitutively overexpressing cell line was unsuccessful, possibly due to growth inhibition caused by ligand-independent activation of stably overexpressed activin receptors. Indeed, an antiproliferative effect of activin has been reported in other systems (12–14, 40). For this reason, we generated an inducible cell line in which type IB and type II activin receptors can be overexpressed just prior to analysis. In this stable cell line, KAR6, the basal level of expression of ActRIB and ActRII is similar to that observed for normal K562 cells but can be induced upon induction by IPTG. We first examined ligand-induced receptor association in this inducible cell line. In KAR6 cells cultured in the absence of ligand, no detectable complex exists between the two types of receptor. Upon stimulation by activin, the type I and II receptors form a heteromeric complex. This is in agreement with observations in mink lung cells transiently transfected with ActRIB and ActRIIB where an increase in complex formation is observed following activin stimulation (3). However, in our inducible stable cell line, no ligand-independent complex formation between the two receptors is observed. Transient overexpression of the activin receptors (type I and II) generates high levels of receptor and leads to ligand-independent association and activation of the receptors (3, 35). In our system we have demonstrated a strict ligand dependence on heteromerization between the type I and II activin receptors. It will be interesting to determine the stoichiometry of these receptor complexes. Indeed, it has been recently shown that TGF-β induces an heterotetrameric complex between its two types of receptors (15, 36, 39).

In this system, the level of overexpression of ActRIB and ActRII is much lower than that observed in transient-transfection systems and is therefore a closer reflection of physiological situations. Our results indicate that the association between ActRIB and ActRII is very rapidly induced within 1 min following ligand stimulation, sustains for 15 min, and then begins to slowly decrease after 30 min. Hence, the first step in the mechanism of action of the activin receptors involves transient oligomerization of different subunits. This is similar to the mechanism described for the TGF-β receptor (37) and to a larger extent to that observed for the tyrosine kinase and hematopoietic receptors (9, 33). Ligand-induced oligomerization of receptor subunits may, therefore, represent a general mechanism of action for members of distinct superfamilies of growth factors.

[^{32}P]orthophosphate metabolic labeling in IPTG-induced KAR6 cells revealed that ActRII is constitutively phosphorylated and activin stimulation did not increase its level of phosphorylation, which is in agreement with results published previously by us and others. We were unable to detect any phosphorylation of ActRIB, possibly due to strong serine/threonine phosphatase activities and to the fact that the stable cell line overexpresses only a limited number of receptors which are probably not sufficient to allow the detection of its phosphorylated residues.

In KAR6 and KAR13 cells, neither transcriptional activation of the 3TPLux reporter construct nor hemoglobin synthesis is affected by IPTG induction alone, in the absence of activin. When IPTG-treated KAR6 cells are stimulated with activin, these two responses are greatly enhanced compared to that observed in normal K562 cells. In IPTG-treated KAR13 cells, where ActRIB only is overexpressed, activin can also enhance the 3TPLux response compared to K562 cells. This suggests that in the type I-type II receptor heterocomplex, ActRIB is the signaling unit, activating downstream targets, while ActRII will recruit the ligand and transphosphorylates the type I receptor with its kinase domain. We, therefore, conclude that our inducible model system is physiologically relevant for the study of activin receptor signaling mechanisms and subsequent downstream biological responses.

We then used these two stable cell lines to investigate the role of activin on cell proliferation. Activin indirectly stimulates proliferation of erythroid progenitor cells through an interaction with both monocytes and T lymphocytes (26). Indeed, activin has no effect on DNA synthesis or cell proliferation in purified erythroid progenitor cells depleted of accessory cells (26). In K562 cells, activin can induce a transient arrest in G_1 phase after 24 h, which is associated with dephosphorylation of the retinoblastoma protein, but the cells recover after 3 days. At day 5, DNA synthesis in activin-treated cells was reported to be greater than that in controls (25). In the present study, a clear decrease in cell proliferation persisted even after 4 days of activin treatment of both KAR6 and KAR13 cells, and the magnitude of this inhibitory effect increased with time. A similar antiproliferative effect of activin was also observed in gonadal cells (12), thymocytes (13), and hepatocytes (40) and may represent a general effect of activin on its target tissues.

Finally, we investigated the antagonistic effect of inhibin on activin-induced erythroid differentiation. Activins (ββ) and inhibins (αβ) share a common subunit, and inhibin antagonizes activin effects in some but not all of its target tissues. In K562 cells, inhibin can suppress activin-induced erythroid differentiation in a dose-dependent manner (41). Inhibin does not by itself affect erythroid differentiation, as measured by the lack of effect on activin receptor heteromerization, 3TPLux activity, hemoglobin synthesis, and cell proliferation in both wild-type K562 cells and our inducible cell lines. In human hepatoma cells (HepG2), cross-linking experiments suggested that inhibin binds to the type II receptor through its β subunit, but its α subunit apparently fails to associate with the type I receptor. Therefore, the inhibin heterodimer could act as a dominant-negative regulator of complex formation between the two types of activin receptors and the activin dimer (38). Our results show that overexpression of ActRII and ActRIB, but not ActRIB alone, blocks the inhibitory effects of inhibin on activin-induced receptor heteromeric complex formation, erythroid differentiation, and cell proliferation (KAR6 cells). However, in the absence of high levels of ActRII (K562 and KAR13 cells), inhibin fully antagonizes all of the above-mentioned activin-induced effects. This is consistent with the hypothesis that the antagonistic effects of inhibin in these cells are mediated via competition for the type II receptor as described for hepatocytes but is not sufficient to explain the antagonism. Indeed, in IPTG-treated KAR6 cells the antagonistic effect of inhibin on activin-induced biological responses cannot be restored even when using a large molar excess of inhibin. This suggests the involvement of another component in the mediation

of this antagonistic effect of inhibin. This component may be present in limiting concentrations that are overwhelmed by high levels of ActRII and ActRIB in the KAR6 cells. Our cross-linking experiments revealed the presence of such inhibin binding subunit, which may represent the first visualization of an inhibin receptor. We, therefore, propose a model for the antagonistic effect of inhibin on activin functions in which inhibin competes with activin for the type II activin receptor. Given that inhibin has a lower affinity than activin for ActRII, the recruitment of an inhibin receptor might stabilize the complex formed by inhibin and ActRII, thus blocking the activin effect. Finally, we cannot exclude the possibility that this inhibin receptor will transduce some inhibin-specific responses which remain to be determined.

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