Activin receptor-like kinase-2 inhibits activin signaling by blocking the binding of activin to its type II receptor

Nina Renlund, Francis H O'Neill¹, LiHua Zhang, Yisrael Sidis² and Jose Teixeira

Vincent Center for Reproductive Biology, Massachusetts General Hospital and Harvard Medical School, Thier 913, 55 Fruit Street, Boston, Massachusetts 02114, USA

¹Pediatric Surgical Research Laboratories and ²Reproductive Endocrine Unit, Massachusetts General Hospital and Harvard Medical School, Boston, Massachusetts 02114, USA

(Correspondence should be addressed to J Teixeira; Email: teixeira@helix.mgh.harvard.edu)

Abstract

Activin receptor-like kinase-2 (Alk2) has been shown to be a promiscuous type I receptor for the transforming growth factor β (TGF β) family of growth and differentiation factors, such as activin, bone morphogenetic proteins, and Müllerian inhibiting substance (MIS). We have studied the putative role of Alk2 in activin signaling using MA-10 cells, a mouse transformed Leydig cell line, in which endogenous expression of cytochrome P450 c17 hydroxylase/C17-20 lyase mRNA is inhibited by both MIS and activin A. Overexpression of Alk2 in MA-10 cells inhibited the activation of the activin-responsive CAGA-luciferase reporter and, conversely, transfection of siRNA for Alk2 increased the response. In contrast, overexpression of the MIS type II receptor in MA-10 cells

increased the activin-mediated induction of CAGA-luciferase approximately fivefold, which we hypothesized occurs by MIS type II receptor sequestering endogenous Alk2. Binding experiments with ¹²⁵I-labeled activin show that the underlying mechanism of Alk2-mediated inhibition of activin signaling involves Alk2 blocking the access of activin to its type II receptor, which we show can bind Alk2 in the absence of ligand. These results show that the complement of other type I receptors in addition to the ligand-specific type I receptor can provide an important mechanism for modulating cell-specific responses to members of the TGF β family.

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Introduction

The transforming growth factor β (TGF β) family of cytokines signals through conserved type I and type II single transmembrane, serine/threonine kinase receptors that dimerize upon ligand binding. Based upon their downstream signaling components, the ligands in this family fall into two distinct subsets: the TGF β /activin group and the bone morphogenetic proteins (BMP)/Müllerian inhibiting substance (MIS) group (Massague & Chen 2000). In the TGF β /activin group, ligand specificity is determined by the type II receptor, which recruits the appropriate type I receptor into the complex. The ligand-bound type II receptor phosphorylates the type I receptor, activating its latent kinase for subsequent downstream signaling via intracellular Smad proteins. The BMP/MIS group appears to initially bind ligand through either the type I receptor or the type II receptor but still requires the type II for activation of the type I and downstream signaling. Smads fall into three different classes, receptor-regulated R-Smads, inhibitory Smads, and the common Smad4. The R-Smads2 and 3 are phosphorylated by the TGF β /activin type I receptors, whereas the R-Smads1, 5, and 8 are phosphorylated by the BMP type I receptors. These phosphorylated Smads then interact with the common Smad4 to form heteromeric complexes that translocate to the nucleus and

effect their respective activities by binding to Smad-responsive DNA elements, alone with a relatively loose specificity or in a supercomplex with cofactors that can modulate ligand-specific gene expression such as FAST-1 and CBP/p300.

How cells respond specifically to a family of greater than 30 secreted members with only five type II receptors and seven type I receptors is still not well understood. Some of the type I receptors have shown a remarkable degree of promiscuity for both ligand and type II receptor, which suggests a mechanism whereby their combination provides an additional level of specificity (Piek *et al.* 1999). For example, activin receptor-like kinase-2 (Alk2) was originally thought to be an activin type I receptor because it could bind activin in the presence of the activin type II receptors (Attisano *et al.* 1993, Tsuchida *et al.* 1993). Subsequent studies showed that Alk2 could not transduce activin-mediated signaling but could transduce both BMP and MIS signaling (ten Dijke *et al.* 1994, Clarke *et al.* 2001, Visser *et al.* 2001). However, the functional significance of Alk2-binding activin type II receptors was not well resolved.

Both MIS and activin can inhibit steroidogenesis in testicular Leydig cells, which are the predominant source of testosterone in males (Lin *et al.* 1989, Mauduit *et al.* 1991, Laurich *et al.* 2002). Using the MA-10 mouse Leydig tumor cell line, we investigated the functional significance of Alk2 in activin-mediated

inhibition of testosterone synthesis. Recent studies by others have led to speculation that the differential expression of Alk1 and Alk2 may represent a mechanism for specifying cell-specific responses to TGF β (Oh *et al.* 2000, Desgrosellier *et al.* 2005). Here, we show evidence that Alk2 inhibits activin signaling by preventing activin binding to its type II receptor.

Experimental procedures

Chemicals and reagents

Waymouth's MB 752/1, horse serum, and gentamicin were obtained from Invitrogen Life Technologies Inc. Protein G Sepharose 4 Fast Flow was purchased from Amersham Biosciences. Monoclonal myc-tagged and HA-tagged antibodies were purchased from Covance Inc. (Berkeley, CA, USA). His-tagged polyclonal antibody, phospho-Smad2, and Smad2 antibody were obtained from Cell Signaling (Danvers, MA, USA). Radionuclides were purchased from Perkin-Elmer (Boston, MA, USA). Recombinant human MIS was prepared from Chinese hamster ovary (CHO) cells stably transfected with a construct of the human MIS gene and purified from serum-free media by serial carbohydrate affinity and ion exchange chromatography (Lorenzo *et al.* 2002). Activin A and the BMPs were purchased from R&D Systems (Minneapolis, MN, USA). The CAGA-Luc and BMP-responsive element (BR)-Luc reporter plasmids were provided by Dr ten Dijke (The Netherlands Cancer Institute, The Netherlands).

Cell culture

MA-10 mouse Leydig tumor cell line (Ascoli 1981), originally a gift from Dr Mario Ascoli (University of Iowa, Iowa City, IA, USA), was cloned and cultured in Waymouth's MB 752/1 supplemented with 15% horse serum, 20 mM HEPES (pH 7.4), penicillin/streptomycin, and 50 μ g/ml gentamicin (Laurich *et al.* 2002). Cells were maintained at 37 °C with 5% CO₂ in a humidified incubator. Transfections were performed using either Fugene6 (Roche Molecular Biochemicals) or TransIT-LT1 (Mirus Bio Corporation, Madison, WI, USA) lipid reagents the day after plating, and the experiments were performed 2 days post-transfection. MA-10 cells were normally transfected with 30–50% efficiency.

Western blotting

Eluates were run on precast 4–12% NuPAGE Bis-Tris gels with 3-(N-morpholino) propane sulfonic acid (MOPS) buffer (Invitrogen) followed by transfer to nitrocellulose membrane. Membranes were blocked for 1 h in 5% non-fat dry milk in tris-buffered saline (TBS)–0.1% Tween 20, incubated with a phospho-Smad2-specific antibody or total Smad2 overnight at 4 °C according to the manufacturer's instructions and detected with a horseradish peroxidase (HRP)-conjugated anti-rabbit IgG followed by enhanced chemiluminescence (Pierce Biotechnology Inc., Rockford, IL, USA).

Northern blotting

Total RNA was collected from treated cells using TRIzol (Invitrogen) reagent according to the manufacturer's instructions and quantitated by absorbance at 260 nm. RNA was separated by electrophoresis, blotted onto nylon membranes, and probed with a cytochrome P450 (Cyp17) antisense riboprobe as described (Laurich *et al.* 2002). Blots were subsequently reprobed with a human actin cDNA probe. Quantitation of the signal intensity was performed with a Fuji BAS1800 Phosphorimager. Northern results were analyzed by ANOVA on repeated measurements followed by the Bonferroni multiple comparison test with GraphPad Software, San Diego, CA, USA.

Ligand binding and cross-linking

MA-10 cells cultured in 100 mm dishes and transfected with HA-tagged type I receptors and myc-tagged activin type II receptor were washed before addition of Krebs–Ringer buffer (20 mM HEPES (pH 7.5) and 5 mM MgSO₄) with 0.5% BSA and treated with activin labeled with ¹²⁵I (kindly provided by Dr Alan Schneyer, Massachusetts General Hospital) for 1 h followed by cross-linking using 1 mM disuccinimidyl suberate (Pierce Biotechnology Inc.) for an additional 2 h at 4 °C. The reaction was stopped by adding 1 M Tris (pH 7.5) to a final concentration of 20 mM. Cells were washed in cold PBS and protein extracts were collected in lysis buffer (20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, and 1 mM β -glycerol phosphate) containing protease inhibitor cocktail (Roche). Protein was quantified using the Bradford method according to the manufacturer's instructions (Bio-Rad Laboratories). One milligram of total protein was incubated with a mouse monoclonal myc antibody overnight at 4 °C. The following day, immunoprecipitates were incubated with protein G-sepharose for 2 h at 4 °C, washed three times, and eluted in loading buffer containing dithiothreitol by heating at 100 °C for 5 min. Eluates were run on precast 4–12% NuPAGE Bis-Tris gels with MOPS buffer (Invitrogen) and dried. Results were detected by autoradiography using intensifying screens.

Co-immunoprecipitations

COS-7 cells cultured in DMEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and penicillin/streptomycin were transiently transfected with tagged receptor constructs and enhanced green fluorescent protein (EGFP)-N1 using TransIT-LT1 transfection reagent (Mirus). After 24-h incubation, the cells were washed with PBS and proteins were isolated in 400 μ l mammalian protein extraction reagent (Pierce). For immunoprecipitation, 200 μ l protein lysate was incubated with a myc-tagged polyclonal (Abcam, Cambridge, MA, USA) or His-tagged polyclonal antibody (Cell Signaling) at 4 °C overnight with mixing. Immune complexes were collected by addition of 20 μ l Protein G Sepharose 4 Fast Flow (Amersham Biosciences) and mixed for 1 h at 4 °C. The pellet was washed thrice in PBS

and the complex eluted by boiling in 20 µl NuPAGE lithium dodecyl sulfate (LDS) sample buffer (Invitrogen) and separated on a 4–12% NuPAGE Bis–Tris gel in MOPS buffer (Invitrogen) under non-reducing conditions. Proteins were transferred to a nitrocellulose membrane and blocked in 5% milk in TBS–T before incubation with mouse monoclonal HA-antibody 1:1000 (Covance). The membranes were washed before incubation with HRP-conjugated secondary antibody and the proteins were visualized with SuperSignal West Pico Chemiluminescent substrate (Pierce).

Luciferase experiments

Cells were plated on day 0 in 24-well plates and transfected with constant total concentration of DNA in a given experiment on day 1. Cells were transfected with an activin/TGFβ-responsive promoter, firefly luciferase reporter construct, CAGA-luc (Dennler *et al.* 1998), or the BMP-responsive promoter/reporter, BRE-luc (Korchynskyi & ten Dijke 2002), in conjunction with a thymidine kinase (TK) promoter–*Renilla* luciferase reporter to control for transfection efficiency. The following day, cells were either untreated or treated overnight as indicated. Cell lysates were harvested on day 3 and luciferase activity was measured using the Dual Luciferase system from Promega. Experiments were performed in triplicate and repeated at least twice more. In the type II receptor-transfection experiments, CAGA and BRE promoter-driven luciferase activity was normalized to the *Renilla* luciferase activity, averaged, and plotted. Luciferase results were analyzed by one-way or two-way ANOVA, as appropriate, on the repeated measurements followed by the Bonferroni *post hoc* test with GraphPad Software. In the Alk-transfected experiments where *Renilla* luciferase was also regulated, a representative of at least three experiments is shown.

siRNA experiments

MA-10 cells were plated in 96-well plates at a density of 10 000 cells/well. The following day, cells were transfected with constructs for the activin type II receptor A (ActRIIA), CAGA-luc, and phRL-cytomegalovirus (CMV) *Renilla* luciferase reporter using TransIT-LT1 and SiControl Non-targeting siRNA #3 D-001210-03-05 (Dharmacon Inc., Lafayette, CO, USA), or siGENOME SMARTpool M-042047-00-0005, mACVR1, NM_007394 (Dharmacon) using TransIT-TKO. On the day after transfection, cells were treated with activin for 24 h and the luciferase activity was recorded using the Dual Luciferase system (Promega). Significance was assigned at $P < 0.05$ using the Student *t*-test.

Results

Activin inhibits Cyp17 expression in MA-10 Leydig cells

Both activin and MIS have been shown to inhibit testosterone synthesis in primary cell cultures and *in vivo* respectively (Lin

et al. 1989, Trbovich *et al.* 2001). In MA-10 Leydig cells, we have previously shown that MIS inhibits testosterone synthesis, at least in part, by inhibiting the expression of cytochrome P450 C17 lyase/17α-hydroxylase (Cyp17; Laurich *et al.* 2002). In order to determine whether these cells could also be used to study endogenous activin signaling, MA-10 cells were treated with activin both in the absence and in the presence of 8Br-cAMP, an inducer of Cyp17 expression (Fig. 1). We show in this representative northern blot experiment that 5 ng/ml activin A inhibits Cyp17 mRNA expression in resting MA-10 cells and significantly reduces Cyp17 expression in cAMP-stimulated cells. The degree of inhibition was consistently less than that observed with MIS. However, MIS was added at a concentration of 5 µg/ml, the concentration that we normally need to use to observe complete regression of the Müllerian duct in organ culture (Donahoe *et al.* 1977), which is 1000-fold higher than the concentration of activin used. Quantitative analyses of several northern blots with a phosphorimager show that the inhibition of Cyp17 mRNA expression by activin is best observed when the cells have been stimulated with cAMP. These results indicate that MA-10 Leydig cells are responsive to activin signaling.

Figure 1 Activin inhibits the expression of Cyp17. (A) MA-10 cells were treated overnight with vehicle as control (NT), 50 µM 8Br-cAMP (cAMP), 5 ng/ml activin A (A) or both (AC), as well as with 5 µg/ml MIS (M) and MIS and cAMP (MC). Total RNA was collected and analyzed by northern blot for Cyp17 mRNA expression. Blots were then reprobbed for actin. Shown is a representative blot. (B) A phosphorimager was used to quantitate signal intensities from three separate northern blot experiments, which were normalized to actin and control-treated cells then plotted. Error bars represent S.E.M.; in cAMP-treated cells, Cyp17 expression was significantly induced when compared with untreated (NT), * $P < 0.01$; activin-treated (ActA), ** $P < 0.01$; and activin- and cAMP-treated (A+C), ** $P < 0.01$.

Alk2 inhibits activin-induced signaling

Activin and MIS are thought to activate the Smad2/3 and Smad1/5/8 pathways respectively. However, in light of the observation that both activin and MIS inhibit the expression of Cyp17 in MA-10 cells, we speculated whether activin and MIS might share a common downstream signaling component. Both activin type II receptors and MISRII bind Alk2, which was once thought to be a functional type I receptor for activin and is considered one of the putative MIS type I receptors (Clarke *et al.* 2001, Visser *et al.* 2001). Therefore, we investigated whether Alk2 might be playing a role in activin signaling in MA-10 cells using the activin-responsive CAGA-luc reporter (Fig. 2A). Transfected Alk2 inhibited CAGA-luc activity to a level that was approximately half that of control-transfected cells. In contrast, transfection of Alk4, the activin type I receptor, resulted in a 15-fold increase in CAGA-Luc activity. Similar results were observed with cells stimulated with activin A (Fig. 2B). Addition of 5 ng/ml activin A to control-transfected cells caused a nearly 14-fold increase in CAGA-Luc activity over untreated cells. CAGA-Luc reporter activity after activin treatment of Alk2- and Alk4-transfected cells was reduced to one-third and induced by nearly

threefold respectively, when compared with that observed in activin-treated, control-transfected cells. In contrast, cotransfection of Alk2 with the MIS/BMP-responsive BRE-Luc reporter induced luciferase activity approximately threefold (Fig. 2C and D). Overexpression of Alk2 enhanced BRE-Luc expression (Fig. 2D), as well as that of control TK and CMV Renilla luciferase reporters (data not shown), in both untreated and BMP2-treated cells, ensuring that the Alk2-mediated transcriptional inhibition was not general but likely particular to CAGA-Luc. These results indicate that in MA-10 cells, Alk2 acts as a BMP type I receptor and can inhibit downstream signaling by activin, while enhancing downstream signaling by MIS. Therefore, activin and MIS signaling through Alk2 could not be a common mechanism for inhibition of Cyp17 expression. This left the possibility that, while Alk2 might be involved in MIS signaling, with activin, Alk2 plays a non-signaling role.

Inhibition of Alk2 enhances activin signaling

To better examine the role of Alk2 in activin signaling, we used an siRNA for Alk2 to inhibit expression of endogenous Alk2 expression. Transfection of siAlk2 into MA-10 cells

Figure 2 Alk2 inhibits activin-induced transcription. MA-10 cells were transfected in triplicate with the CAGA-luc and TK-Renilla luciferase reporters (A and B) and cotransfected with either a control plasmid (C), or Alk2, Alk3, or Alk4 expressing plasmids as indicated. The following day, cells were either untreated (A) or treated overnight with 5 ng/ml activin A (B). Luciferase activity was measured and normalized to the TK-Renilla. MA-10 cells were also transfected as above with the BRE-luc reporter (C and D) and either the control plasmid (C), or Alk2, or Alk4. The next day, cells were either untreated or treated with activin A (C) or BMP2 (D). The results shown are representative of three experiments performed in triplicate and error bars represent s.e.m. CAGA-luciferase activity was significantly inhibited in cells transfected with Alk2 and treated with activin when compared with control-transfected cells treated with activin, * $P < 0.001$.

resulted in almost a doubling of activin-induced luciferase activity compared with when a control siRNA was used (Fig. 3). These results indicate that there might be a constant inhibitory effect of endogenous Alk2 on activin signaling.

Smad2 phosphorylation is inhibited by Alk2

Activin binding to its heteromeric receptor complex induces a conformational change that triggers the activation of the latent kinase domain of the type I receptor for subsequent downstream signaling via the Smad2/3. Therefore, we investigated whether overexpression of Alk2 affected the phosphorylation of Smad2. Because we expected that high levels of overexpression would be needed to observe an effect on endogenous Smad2 phosphorylation, we used COS cells transfected with either control plasmid or an Alk2 expression construct. The amount of phosphorylated Smad2 was determined by western blot analysis with an antibody specific to phosphorylated Smad2 (pSmad2; Fig. 4A). Overexpression of Alk2 inhibited the basal phosphorylation in these cells, suggesting that Alk2 inhibited activin signaling upstream of Smad2 phosphorylation. Addition of activin did not affect the level of pSmad2 phosphorylation above basal levels in COS cells, precluding our ability to assay the effect of Alk2 overexpression on pSmad2 in the presence of added activin. These basal levels of pSmad2 are presumably due to endogenous expression of either activin or TGF β . Western blot analyses were also performed to ensure that overexpression of Alk2 was not having an indirect effect on activin signaling by inducing expression of Smad7, the inhibitory Smad (data not shown). We have also performed these experiments in MA-10 cells (Fig. 4B). Although expression of the transgene is not nearly as high as it is in COS cells, we

Figure 3 Inhibition of Alk2 increases activin signaling. MA-10 cells were transfected with ActRIIA, CAGA-luc, and phRL CMV luciferase in combination with an siRNA for Alk2 or a control siRNA. The cells were treated for 24 h with 2.5 ng/ml activin and the lysates were measured for luciferase activity. The results shown represent three independent experiments performed in triplicates, and error bars represent s.e.m., * $P < 0.05$.

Figure 4 Smad2 phosphorylation is reduced with overexpression of Alk2. Cells were transfected with a control DNA plasmid or with an Alk2 expression construct for 24 h and thereafter put in serum-free media before treatment with 25 ng/ml activin for 50 min. Cell lysates were prepared with phosphatase inhibitors and subjected to PAGE and western analysis with a pSmad2-specific and Smad2 antibody. The blots are representative of at least three independent experiments.

were still able to observe a noticeable decrease in pSmad2 phosphorylation.

MISRII expression enhances CAGA-Luc reporter activity

MISRII has previously been shown to be specific for the MIS ligand (Mishina *et al.* 1996) and that downstream signaling by the MISRII occurs through the BMP group of type I receptors and Smads, including Alk2 (Gouedard *et al.* 2000, Clarke *et al.* 2001, Visser *et al.* 2001, Jamin *et al.* 2002). Therefore, we attempted to exploit the ability of MISRII to interact and sequester Alk2 as a mechanism to induce activin signaling. Although overexpression of the MISRII in MA-10 cells raised the basal level of BRE reporter activity by approximately twofold (data not shown), we show that it had no significant effect on the activin-, MIS-, or BMP-induced activation of the BRE-Luc over the control plasmid (Fig. 5A). With either the control plasmid or the MISRII expression construct, activin inhibited BRE reporter activity by at least 50%, while addition of MIS or BMP2 increased reporter activity two- to threefold respectively. In contrast, MISRII overexpression enhanced the activin-induced CAGA-Luc activation by approximately fivefold enhancement of luciferase activity (Fig. 5B). Neither MIS nor BMP had any effect on this reporter.

In order to explore the enhanced induction of the activin-responsive promoter by MISRII expression further, luciferase experiments were performed with a wide range of activin concentrations (Fig. 5C). Cotransfection of MA-10 cells with the MISRII increased CAGA reporter activity approximately twofold at 2 ng/ml activin A over cotransfection with a control plasmid; 25 ng/ml caused more variable increases that averaged approximately threefold higher than with the control. MISRII-induced activin signaling was also observed in HepG2 cells, which were tested to assess whether the phenomenon was specific to MA-10 cells (data not shown). To determine whether MISRII receptor kinase activity was required for the induced CAGA reporter activity, MA-10 cells were also transfected with a mutated form of the receptor

Figure 5 MISRII enhances activin-induced transcription. (A) MA-10 cells were transfected with the BMP-responsive BRE-firefly luciferase reporter, a thymidine kinase (TK) promoter-*Renilla* luciferase control, and either a control or the MISRII expression plasmid. On the following day, cells were treated overnight with boiled MIS as a control (C), 25 ng/ml activin A (A), 5 µg/ml MIS (M), and 25 ng/ml BMP2 (B). Firefly luciferase activity was assayed in cell lysates, normalized to *Renilla* luciferase, and normalized again to control-treated activity. The mean inductions of a representative experiment are shown with error bars representing s.e.m. (B) Cells were cotransfected with the activin-responsive CAGA-firefly luciferase reporter, treated, and analyzed as in A. (C) Cells were cotransfected with the indicated expression constructs for control, MIS type II receptor (MISRII), a kinase-inactive mutant MISRII (K228R), and activin type II receptor-A (ActRIIA). On the following day, cells were either untreated or treated overnight with activin A (1, 2, 5, and 25 ng/ml). Fold induction by activin was calculated by normalizing firefly luciferase activity to *Renilla* luciferase and then normalizing again to control cells treated with 25 ng/ml activin, which was set to 100%. The average of three experiments performed in triplicate is shown, and error bars represent s.e.m. Significantly different values from the 25 ng/ml control are indicated, * $P < 0.05$, ** $P < 0.001$.

with an inactive kinase domain (K228R; Clarke *et al.* 2001). Similar experiments with BMPRIIA-transfected cells did not significantly induce CAGA-luciferase activity over that of control-transfected cell, suggesting that the phenomenon was specific to MISRII (data not shown). Although the mean induction was lower in cells treated with 25 ng/ml activin than in cells transfected with the plasmid expressing the native MISRII, the induction of CAGA reporter activity was still significantly higher than that of control-transfected cells (Fig. 5C). These results suggested that MISRII expression in MA-10 Leydig cells enhances activin signaling in the absence of MISRII signaling. In contrast, transfection of MA-10 cells with the activin type II receptor expression construct (ActRIIA) showed that, although the basal level of CAGA reporter activity was sixfold higher than that of control-

transfected cells and 1 ng/ml activin A did increase the response by twofold, further increasing concentrations of activin were not able to induce the relative luciferase activity significantly (Fig. 5C). Similar results were observed with ActRIIB (data not shown).

Alk2 blocks the binding of activin to its type II receptor

One of the possible mechanisms that may be used by Alk2 to downregulate activin signaling prior to Smad2 phosphorylation would be by interfering with the productive binding of activin to the activin type II receptor. We investigated this possibility using binding assays with radiolabeled activin A (Fig. 6). MA-10 cells were transfected with ActRIIA and either with a control plasmid or with expression constructs for

Figure 6 Alk2 inhibits the binding of activin to the activin type IIA receptor. MA-10 cells were transfected with myc-tagged ActRIIA alone or with the indicated type I receptors. Two days following transfection, cells were incubated with ^{125}I -activin A and cross-linked before lysis and immunoprecipitation with a myc monoclonal antibody. The immunoprecipitations were analyzed by PAGE, and the gel was dried and exposed to X-ray film with intensifying screens. The activin-receptor type I, type II, and both complexes are indicated on the left.

the TGF β type I receptor, Alk5; the activin type I receptor, Alk4; and Alk2. Cells that were transfected with ActRIIA alone or together with Alk5 showed two cross-linked bands with the radiolabeled activin representing what is believed to be two different glycosylated forms of the activin type II receptor (Harrison *et al.* 2003). Cotransfection with Alk4 showed an additional band that represents activin cross-linked to the shorter type I receptor. Faint cross-linked activin bands were observed in cells that were cotransfected with Alk2, suggesting that Alk2 inhibited activin signaling by blocking activin binding to its receptor. The highest molecular weight band likely represents activin cross-linked to a complex of ActRII and either endogenous or transfected Alk4.

Alk2 binds ActRIIA in the absence of ligand

We have shown above that Alk2 inhibits activin signaling and transfection of the MISRII cDNA enhanced activin signaling by a mechanism that we speculate involves the sequestering of Alk2 away from activin. Furthermore, in Fig. 6, we showed that Alk2 cotransfection inhibited binding of iodinated activin to the ActRIIA receptor. These results have led us to hypothesize that Alk2 inhibits activin signaling by binding the activin type II receptor and blocking access of the ligand to its binding site. Therefore, Alk2 would have to bind ActRIIA in the absence of ligand. In order to test this possibility, we cotransfected COS cells with ActRIIA or MISRII and either Alk2 or green fluorescent protein (GFP) as a negative control and co-immunoprecipitated the complexes (Fig. 7). Western blot analysis of the immunoprecipitated complexes shows that Alk2 is co-immunoprecipitated with both ActRIIA and MISRII but not GFP, and that the immunoprecipitated complexes occur in the absence of ligand. Additional experiments were performed with added

Figure 7 Alk2 interacts with ActRIIA and MISRII independently of ligand. COS cells were cotransfected with 6xHis-tagged ActRIIA, HA-tagged Alk2, myc-tagged MISRII, and GFP expression constructs as indicated. Lysates were prepared from the cells, immunoprecipitated with an anti-HIS or anti-myc antibody, and subjected to western blot analysis with an anti-HA antibody. Cell lysate from Alk2/GFP-transfected cells before immunoprecipitation is shown as a control for the Alk2 migration distance.

MIS and BMP, but these ligands did not interfere with the ActRIIA/Alk2 co-immunoprecipitations (data not shown).

Discussion

Alk2 was originally described as an activin (Attisano *et al.* 1993, Tsuchida *et al.* 1993) or TGF β type I (Ebner *et al.* 1993) receptor based on its binding in a complex with the respective type II receptor. Subsequent studies showed that Alk2 also bound BMP7 and the BMP type II receptors, suggesting that Alk2 may be a shared type I receptor (ten Dijke *et al.* 1994, Yamashita *et al.* 1994) or may only be involved in BMP pathway signaling (Macias-Silva *et al.* 1998). Here, we show evidence that Alk2 inhibits activin-mediated downstream signaling by interfering with the binding of activin to its type II receptor. We speculate that Alk2 expression in a cell may act not only to mediate MIS/BMP pathway signaling but also to suppress TGF β /activin pathway signaling.

In the presence of MIS ligand, Alk2 and MISRII initiate BMP pathway-specific signaling (Clarke *et al.* 2001, Visser *et al.* 2001), but the interaction of Alk2 with MISRII and its subsequent sequestration in the absence of ligand might also prove to be a physiological mechanism to induce activin signaling. The molecular details of such a mechanism remain to be elucidated. MISRII can also signal through Alk3 (Jamin *et al.* 2002) and Alk6 (Gouedard *et al.* 2000); our studies with Alk3, however, show that overexpression of Alk3 does not appear to affect activin signaling. Additionally, increasing the amount of activin added to cells transfected with the MISRII expression construct but not the ActRIIA expression construct led to increasing CAGA-luc expression, suggesting that overexpression of ActRIIA is not sufficient to overcome the inhibitory effect of Alk2 and/or other type I receptors.

MISRII has previously been shown to interact in a ligand-independent manner with Alk5 in transfected CHO cells (Gouedard *et al.* 2000). In that report, MISRII interaction with Alk6 was enhanced with MIS ligand and neither Alk2 nor 3 could be immunoprecipitated with MISRII in the presence or absence of MIS ligand. This raises the possibility that another factor that might be absent in CHO cells but present in MA-10 may be facilitating MISRII interaction with Alk2 or conversely, that CHO cells express an inhibitor of MISRII and type I heterodimerization. The latter situation would be similar to that shown with the pseudoreceptor, BAMBI (BMP and activin membrane-bound inhibitor), which binds to type I receptors and inhibits downstream signaling by preventing formation of active receptor complexes (Onichtchouk *et al.* 1999). Early studies to identify the type I receptor partner for MISRII using yeast two-hybrid assays (Wang *et al.* 1996) showed that the cytoplasmic domain of the rat MISRII interacted with those of all the type I receptors tested (data not shown). In support of these studies, we have recently found that transfected MISRII co-immunoprecipitates with all the type I receptors as well (data not shown). Together, these results indicate that MISRII can interact with multiple type I receptors, and that in cells that express endogenous MISRII, these interactions may be of physiological significance. The finding that inhibition of endogenous Alk2 or its sequestration by another type II receptor induces activin signaling might indicate that differential expression of Alk2 during different times of development could be an important mechanism of regulating activin signaling in steroidogenic and other cells expressing these receptors. Another possibility is that MISRII expression alone may be a mechanism to regulate activin signaling by sequestering Alk2. Additionally, while it would be difficult to assess quantitatively, in the co-immunoprecipitation experiments in Fig. 7, it does appear that MISRII has a greater affinity for Alk2 than does ActRIIA, suggesting that the type I and type II receptors have differing affinities for each other, which would give the cell another mechanism for modulating TGF β family signal transduction. We could also speculate that the relative expression levels and affinities of ActRIIA for Alk2 and Alk4, in either the absence or presence of ligand, may affect the degree of inhibition of activin signaling in a given context.

In summary, we have shown that Alk2 is an inhibitor of activin signaling. A similar role for other TGF β receptor family members may provide yet another mechanism for determining cell-specific responses to individual ligands with a limited repertoire of receptors.

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References

- Ascoli M 1981 Characterization of several clonal lines of cultured Leydig tumor cells: gonadotropin receptors and steroidogenic responses. *Endocrinology* **108** 88–95.
- Attisano L, Carcamo J, Ventura F, Weis FM, Massague J & Wrana JL 1993 Identification of human activin and TGF beta type I receptors that form heteromeric kinase complexes with type II receptors. *Cell* **75** 671–680.
- Clarke TR, Hoshiya Y, Yi SE, Liu X, Lyons KM & Donahoe PK 2001 Mullerian inhibiting substance signaling uses a BMP-like pathway mediated by ALK2 and induces Smad6 expression. *Molecular Endocrinology* **15** 946–959.
- Denhler S, Itoh S, Vivien D, ten Dijke P, Huet S & Gauthier JM 1998 Direct binding of Smad3 and Smad4 to critical TGF beta-inducible elements in the promoter of human plasminogen activator inhibitor-type 1 gene. *EMBO Journal* **17** 3091–3100.
- Desgrosellier JS, Mundell NA, McDonnell MA, Moses HL & Barnett JV 2005 Activin receptor-like kinase 2 and Smad6 regulate epithelial–mesenchymal transformation during cardiac valve formation. *Developmental Biology* **280** 201–210.
- ten Dijke P, Yamashita H, Sampath TK, Reddi AH, Estevez M, Riddle DL, Ichijo H, Heldin CH & Miyazono K 1994 Identification of type I receptors for osteogenic protein-1 and bone morphogenetic protein-4. *Journal of Biological Chemistry* **269** 16985–16988.
- Donahoe PK, Ito Y & Hendren WH 1977 A graded organ culture assay for the detection of Mullerian inhibiting substance. *Journal of Surgical Research* **23** 141–148.
- Ebner R, Chen RH, Shum L, Lawler S, Zioncheck TF, Lee A, Lopez AR & Derynck R 1993 Cloning of a type I TGF-beta receptor and its effect on TGF-beta binding to the type II receptor. *Science* **260** 1344–1348.
- Gouedard L, Chen YG, Thevenet L, Racine C, Borie S, Lamarre I, Jossio N, Massague J & di Clemente N 2000 Engagement of bone morphogenetic protein type IB receptor and Smad1 signaling by anti-Mullerian hormone and its type II receptor. *Journal of Biological Chemistry* **275** 27973–27978.
- Harrison CA, Gray PC, Koerber SC, Fischer W & Vale W 2003 Identification of a functional binding site for activin on the type I receptor ALK4. *Journal of Biological Chemistry* **278** 21129–21135.
- Jamin SP, Arango NA, Mishina Y, Hanks MC & Behringer RR 2002 Requirement of Bmpr1a for Mullerian duct regression during male sexual development. *Nature Genetics* **32** 408–410.
- Korchynskiy O & ten Dijke P 2002 Identification and functional characterization of distinct critically important bone morphogenetic protein-specific response elements in the Id1 promoter. *Journal of Biological Chemistry* **277** 4883–4891.
- Laurich VM, Trbovich AM, O'Neill FH, Houk CP, Sluss PM, Payne AH, Donahoe PK & Teixeira J 2002 Mullerian inhibiting substance blocks the protein kinase A-induced expression of cytochrome p450 17alpha-hydroxylase/C(17-20) lyase mRNA in a mouse Leydig cell line independent of cAMP responsive element binding protein phosphorylation. *Endocrinology* **143** 3351–3360.
- Lin T, Calkins JK, Morris PL, Vale W & Bardin CW 1989 Regulation of Leydig cell function in primary culture by inhibin and activin. *Endocrinology* **125** 2134–2140.
- Lorenzo HK, Teixeira J, Pahlavan N, Laurich VM, Donahoe PK & MacLaughlin DT 2002 New approaches for high-yield purification of Mullerian inhibiting substance improve its bioactivity. *Journal of Chromatography. B, Analytical Technologies in the Biomedical and Life Sciences* **766** 89–98.
- Macias-Silva M, Hoodless PA, Tang SJ, Buchwald M & Wrana JL 1998 Specific activation of Smad1 signaling pathways by the BMP7 type I receptor, ALK2. *Journal of Biological Chemistry* **273** 25628–25636.

- Massague J & Chen YG 2000 Controlling TGF-beta signaling. *Genes and Development* **14** 627–644.
- Mauduit C, Chauvin MA, de Peretti E, Morera AM & Benahmed M 1991 Effect of activin A on dehydroepiandrosterone and testosterone secretion by primary immature porcine Leydig cells. *Biology of Reproduction* **45** 101–109.
- Mishina Y, Rey R, Finegold MJ, Matzuk MM, Jossio N, Cate RL & Behringer RR 1996 Genetic analysis of the Müllerian-inhibiting substance signal transduction pathway in mammalian sexual differentiation. *Genes and Development* **10** 2577–2587.
- Oh SP, Seki T, Goss KA, Imamura T, Yi Y, Donahoe PK, Li L, Miyazono K, ten Dijke P, Kim S *et al.* 2000 Activin receptor-like kinase 1 modulates transforming growth factor-beta 1 signaling in the regulation of angiogenesis. *PNAS* **97** 2626–2631.
- Onichtchouk D, Chen YG, Dosch R, Gawantka V, Delius H, Massague J & Niehrs C 1999 Silencing of TGF-beta signalling by the pseudoreceptor BAMBI. *Nature* **401** 480–485.
- Piek E, Heldin CH & Ten Dijke P 1999 Specificity, diversity, and regulation in TGF-beta superfamily signaling. *FASEB Journal* **13** 2105–2124.
- Trbovich AM, Sluss PM, Laurich VM, O'Neill FH, MacLaughlin DT, Donahoe PK & Teixeira J 2001 Mullerian inhibiting substance lowers testosterone in luteinizing hormone-stimulated rodents. *PNAS* **98** 3393–3397.
- Tsuchida K, Mathews LS & Vale WW 1993 Cloning and characterization of a transmembrane serine kinase that acts as an activin type I receptor. *PNAS* **90** 11242–11246.
- Visser JA, Olaso R, Verhoef-Post M, Kramer P, Themmen AP & Ingraham HA 2001 The serine/threonine transmembrane receptor ALK2 mediates Müllerian inhibiting substance signaling. *Molecular Endocrinology* **15** 936–945.
- Wang T, Li BY, Danielson PD, Shah PC, Rockwell S, Lechleider RJ, Martin J, Manganaro T & Donahoe PK 1996 The immunophilin FKBP12 functions as a common inhibitor of the TGF beta family type I receptors. *Cell* **86** 435–444.
- Yamashita H, ten Dijke P, Franzen P, Miyazono K & Heldin CH 1994 Formation of hetero-oligomeric complexes of type I and type II receptors for transforming growth factor-beta. *Journal of Biological Chemistry* **269** 20172–20178.

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