Cripto Is a Noncompetitive Activin Antagonist That Forms Analogous Signaling Complexes with Activin and Nodal*

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Cripto plays critical roles during embryogenesis and has been implicated in promoting the growth and spread of tumors. Cripto is required for signaling by certain transforming growth factor-β superfamily members, such as Nodal, but also antagonizes others, such as activin. The opposing effects of Cripto on Nodal and activin signaling seem contradictory, however, because these closely related ligands utilize the same type I (ALK4) and type II (ActRII/IIB) receptors. Here, we have addressed this apparent paradox by demonstrating that Cripto forms analogous receptor complexes with Nodal and activin and functions as a noncompetitive activin antagonist. Our results show that activin-A and Nodal elicit similar maximal signaling responses in the presence of Cripto that are substantially lower than that of activin-A in the absence of Cripto. In addition, we provide biochemical evidence for complexes containing activin-A, Cripto, and both receptor types and show that the assembly of such complexes is competitively inhibited by Nodal. We further demonstrate that Nodal and activin-A share the same binding site on ActRII and that ALK4 has distinct and separable binding sites for activin-A and Cripto. Finally, we show that ALK4 mutants with disrupted activin-A binding retain Cripto binding and prevent the effects of Cripto on both activin-A and Nodal signaling. Together, our data indicate that Cripto facilitates Nodal signaling and inhibits activin signaling by forming receptor complexes with these ligands that are structurally and functionally similar.

The transforming growth factor β (TGF-β)3 superfamily contains ~40 structurally related ligands that control a wide range of diverse cellular processes, including proliferation, homeostasis, differentiation, and migration (1, 2). TGF-β ligands exert their biological effects by binding and assembling two types of transmembrane receptors (type I and type II) with intrinsic serine/threonine kinase activities (3, 4). There are five type II receptors and seven type I receptors; therefore, these receptors display varying degrees of promiscuity with regard to the ligands they bind (3). Activin type II (ActRII and ActRIIB) and type I (ALK4) receptors are especially promiscuous and form signaling complexes with activins but also several other superfamily members, including GDF1, GDF3, GDF8 (myostatin), and Nodal (5). In cases where multiple TGF-β ligands share the same receptors, signaling specificity can be achieved via ligand-specific differences, including variable expression patterns, processing of mature ligands from propeptides, stabilities, receptor affinities, susceptibility to extracellular ligand traps, and modulation by co-receptor proteins (3, 4, 6).

As an example, activin and Nodal utilize the same signaling receptors, but their mechanisms of ligand-mediated receptor assembly and use of co-receptor proteins differ significantly. On the one hand, activins initiate signaling by first binding ActRII/IIB with high affinity (7–9) and then recruiting ALK4, resulting in the formation of active signaling complexes (10, 11). By contrast, Nodal lacks intrinsic affinity for ActRII/IIB and ALK4 and requires Cripto (Cripto-1, TDGF1) or a related co-receptor to facilitate its binding to these receptors (12–14). Cripto is a small, GPI-anchored protein that possesses two modular cysteine-rich domains, an epidermal growth factor-like domain that binds Nodal and a CFC domain that binds ALK4 (13, 15, 16). Structure/function studies have shown that Nodal assembles type II and type I receptors only in the presence of Cripto. In fact, disruption of Nodal binding to the epidermal growth factor-like domain or ALK4 binding to the CFC domain completely abolishes Cripto-dependent Nodal signaling (12, 17, 18). Following receptor assembly, propagation of activin and Nodal signals is thought to be essentially the same (i.e. ALK4 is phosphorylated and activated by the constitutively active ActRII/ActRIIB receptor kinase and in turn phosphorylates cytoplasmic Smad2 and Smad3 proteins). Once phosphorylated, Smad2 and Smad3 assemble into complexes together with Smad4 and then migrate into the nucleus, where they interact with transcription factors, co-activators, and co-repressors to regulate transcription of target genes (3).

In contrast to its requirement for Nodal signaling, Cripto has been shown to antagonize activin signaling apparently by preventing activin from assembling its receptors (19). For example, we have demonstrated that Cripto forms complexes with activin-A and ActRII/IIB and reduces activin-A cross-linking to ALK4 (20). This observation led us to propose a model in which Cripto inhibits activin-A signaling by competing with ALK4 for
access to activin-A-ActRII/IIB complexes. Cripto was also shown to bind activin-B, and in this case it was proposed that Cripto inhibits activin-B signaling by forming nonproductive complexes with activin-B and/or ALK4 (21). Although these studies reported distinct inhibitory complexes, they each presented models in which Cripto competitively inhibits activin signaling by blocking receptor assembly. Notably, these models appear to be incompatible with the well documented role of Cripto as a Nodal co-receptor that facilitates receptor assembly. Specifically, it remains unclear how Cripto can form functional signaling complexes with Nodal while inhibiting activin-dependent receptor assembly.

In the present study, we provide evidence supporting a new model that explains the seemingly contradictory effects of Cripto on activin and Nodal signaling. We demonstrate that Cripto is a noncompetitive activin antagonist and that activin and Nodal assemble Cripto-containing receptor complexes that are structurally and functionally similar. We also identify ALK4 mutants that bind Cripto and block its effects on activin-A and Nodal signaling. Together, our data suggest that Cripto functions as a molecular switch that causes cellular responses to activin and Nodal to converge via the formation of analogous signaling complexes.

**EXPERIMENTAL PROCEDURES**

**Materials**—NuPAGE gels and molecular weight markers were obtained from Invitrogen. Recombinant human activin-A was generated using a stable activin-A-expressing cell line generously provided by Dr. J. Mather (Genentech, Inc., South San Francisco, CA) and was purified by Wolfgang Fischer (Peptide Biology Laboratory, Salk Institute, La Jolla, CA). Recombinant mouse Nodal was purchased from R&D Systems. 

125I-Activin-A was prepared using the chloramine T method as described previously (22). Anti-Myc (9E10) monoclonal antibody and protein G-agarose were purchased from Calbiochem. Polyclonal antibodies directed against ALK4 (23) have been described. A polyclonal anti-Cripto antibody (6900) was prepared as described previously (22). Anti-FLAG (M2) antibody and FLAG (M2)-agarose were obtained from Sigma. Anti-phospho-Smad2, anti-Smad2/3, and anti-pan-actin antibodies were purchased from Cell Signaling Technologies (Danvers, MA). Horseradish peroxidase-linked anti-rabbit IgG, anti-mouse IgG, 3,3′,5,5′-tetramethylbenzidine substrate, chemiluminescent substrate (Supersignal™), and the BCA protein assay kit were obtained from Pierce. All DNA constructs used in this study were in the pcDNA3.0 expression vector (Invitrogen).

**Expression Constructs**—The mouse Cripto construct has been described (12) and was a generous gift from Malcolm Whitman (Department of Cell Biology, Harvard Medical School, Boston, MA). The ΔCFC Cripto deletion mutant was generated using a previously described PCR strategy (24), in which the CFC domain was replaced with an in-frame BamHI site (GGATCC) encoding the amino acids Gly-Ser. The kinase-deleted ALK4 constructs (ALK4-trunc) encode the first 206 amino acids of ALK4 and were generated using standard PCR techniques, as previously described (25). Similar standard PCR strategies were used to make kinase-deleted ActRII constructs (ActRII-trunc) and to introduce epitope tags (25, 26). All constructs were confirmed by DNA sequencing.

**Transfection and Detection of Cell Surface Protein Expression in HEK 293T Cells**—HEK 293T cells were grown in 5% CO2 to 40–60% confluence in complete Dulbecco’s modified Eagle’s medium (with 10% bovine calf serum, penicillin, streptomycin, and l-glutamine) on poly-d-lysine-coated wells or plates. Cells were transfected with indicated DNA constructs using Perfectin (Gene Therapy Systems). For Western blotting, cells were solubilized in radioimmune precipitation buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, and 0.1% SDS) containing standard protease inhibitors. SDS-PAGE and electrotransfer to nitrocellulose were carried out using NuPAGE gels and a NOVEX X-cell II apparatus as described previously (26). To detect proteins expressed at the cell surface, intact cells were washed with Hepes dissociation buffer (HDB), blocked with 3% bovine serum albumin/HDB, incubated with anti-FLAG antibody, washed with HDB, and incubated with peroxidase-conjugated anti-mouse IgG. Specific antibody staining was measured using the 3,3′,5,5′-tetramethylbenzidine peroxidase substrate, as described previously (26).

**Luciferase Assays**—Luciferase assays were carried out using the A3-luciferase reporter essentially as previously described (20). The A3-luciferase construct contains three copies of the activin response element from the Xenopus laevis Mix.2 promoter linked to a basic TATA box and a luciferase reporter gene. HEK 293T cells were plated on poly-d-lysine-coated 24-well plates at 1 × 10^5 cells/well and transfected (Perfectin) in triplicate ~24 h later with 500 ng of DNA/well using 10–400 ng of DNA constructs (indicated combinations of pcDNA 3.0 vector, Cripto, wild type (WT) or mutant ActRII-trunc, and WT or mutant ALK4-trunc) and 50 ng FAST2 (FoxH1), 25 ng of A3-luciferase, 25 ng of CMV-β-galactosidase. Cells were treated ~24 h following the transfection and then harvested ~16 h following treatment. Cells were incubated in solubilization buffer (1% Triton X-100, 25 mM glycyglycine (pH 7.8), 15 mM MgSO4, 4 mM EGTA, and 1 mM dithiothreitol) for 30 min on ice, and luciferase reporter activity was measured and normalized relative to CMV-β-Gal activities.

**Smad2 Phosphorylation**—HEK 293T cells were plated on 6-well plates at a density of 2 × 10^5 cells/well. 24 h after plating, cells were transfected with 2 μg of DNA (1 μg of vector and 1 μg of Cripto) using Perfectin. 24 h after transfection, cells were serum-starved overnight prior to treatment. Cells were left untreated or treated for 30 min with the indicated doses of activin-A or Nodal. Cells were harvested by adding 150 μl of ice-cold radioimmune precipitation buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS) supplemented with 50 mM β-glycerol phosphate, 20 mM NaF, and standard protease inhibitors. Fifty μl of 4x SDS-PAGE loading buffer were then added to each sample, and the proteins were separated by SDS-PAGE and blotted to nitrocellulose. Blots were treated with anti-phospho-Smad2 or anti-Smad2/3 antibodies, followed by anti-rabbit IgG antibody conjugated to horseradish peroxidase, and bands were detected using enhanced chemiluminescence.
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Covalent Cross-linking—HEK 293T cells were plated on 6-well plates coated with poly-D-lysine at a density of \( 4 \times 10^5 \) cells/well. Approximately 24 h later, cells were transfected with 2 \( \mu \)g of DNA/well (1 \( \mu \)g of ActRII-Myc, 0.5 \( \mu \)g of ALK4-unmarked and 0.5 \( \mu \)g of WT-FLAG) using Perfectin and then incubated an additional 48 h before harvesting. Covalent cross-linking was performed by first washing cells in HDB (12.5 mM HEPES, pH 7.4, 140 mM NaCl, 5 mM KCl) and then incubating cells with \(^{125}\)I-activin-A in binding buffer (HDB containing 0.1% bovine serum albumin, 5 mM MgSO\(_4\), 1.5 mM CaCl\(_2\)) at room temperature for 4 h. Cells were washed with HDB and incubated in 0.5 mM disuccinimidyl suberate in HDB for 30 min on ice. Cross-linking reactions were quenched with TBS, and cells were solubilized in lysis buffer (TBS containing 1% Nonidet P-40, 0.5% deoxycholate, 2 mM EDTA) with lysates subjected to immunoprecipitation by using anti-Myc, anti-FLAG, or anti-ALK4 antibodies. Immune complexes were then analyzed by SDS-PAGE and autoradiography.

RESULTS

Cripto Reduces Maximal Activin-A-dependent Luciferase Induction to a Level That Converges with That of Nodal—In order to compare the roles of Cripto as a co-receptor for Nodal and an inhibitor of activin-A signaling, 293T cells were transfected with the Smad2-responsive luciferase reporter A3-Lux and FAST2 (FoxH1) in the presence of vector or Cripto and then treated with a range of doses of each ligand. Consistent with previous reports that Nodal requires Cripto as an obligatory co-receptor (14), we did not observe Nodal signaling in the absence of Cripto even at the highest dose of 180 nM Nodal (Fig. 1C). However, in the presence of Cripto, Nodal caused a dose-dependent induction of luciferase with an EC\(_{50}\) of 14 nM and a maximal induction of 20-fold basal (Fig. 1A).

In parallel experiments, we measured the dose-response relationship for activin-A in the absence and presence of Cripto. In the absence of Cripto, maximal doses of activin-A elicited a

![Image](49x451 to 408x733)

**FIGURE 1.** Cripto reduces activin-A efficacy to a level similar to that of Nodal. 293T cells were transfected with A3-lux, FAST2, and CMV-\( \beta \)-galactosidase. Resulting luciferase activities were normalized relative to \( \beta \)-galactosidase activities and are presented as fold induction. A and B, cells were further transfected with 200 ng of empty vector or Cripto plasmid as described under “Experimental Procedures” and were left untreated or treated with the indicated doses of either Nodal or activin-A. C and D, cells were again transfected with the indicated doses of Cripto DNA, and cell surface expression was measured using an intact cell enzyme-linked immunosorbent assay, as described under “Experimental Procedures.”
Cripto reduces activin-A-induced Smad2 phosphorylation to a level similar to that of Nodal. 293T cells were transfected with empty vector or Cripto and treated with the indicated doses of Nodal or activin-A. Resulting cell lysates were subjected to Western blotting (IB) using phospho-Smad2, Smad2, or Cripto antibodies, as described under “Experimental Procedures.”

In summary, three novel conclusions can be drawn from these data measuring the effects of Cripto on Nodal and activin-A signaling: 1) activin-A has a higher intrinsic efficacy than Nodal; 2) Cripto does not change the potency (EC\textsubscript{50}) of activin-A but rather acts in a noncompetitive manner to reduce activin-A efficacy; and 3) the efficacies of activin-A and Nodal converge as Cripto expression increases.

Activin-A forms a complex with ActRII, ALK4, and Cripto. 293T cells transfected with the indicated receptor or Cripto constructs (A–C) or native P19 cells (D) were subjected to \textsuperscript{125}I-activin-A cross-linking, as described under “Experimental Procedures.” Cross-linked complexes were subjected to immunoprecipitation (IP) using antibodies targeting ActRII-Myc (A), ALK4 (B), Cripto-FLAG (C), or native Cripto (D), and immune complexes were resolved via SDS-PAGE and visualized by autoradiography.
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precipitation, we could not rule out the possibility that the labeled bands corresponding to ALK4 and Cripto (Fig. 3A, lane 5) were derived from distinct complexes consisting of activin-A-ActRII-Cripto or activin-A-ActRII-ALK4. This issue was resolved by immunoprecipitation with an anti-ALK4 antibody that resulted in the clear detection of labeled bands corresponding to ActRII, ALK4, and Cripto (Fig. 3B, lane 3). Since ALK4 and Cripto do not bind activin-A in the absence of ActRII (20), this result demonstrates that activin-A, ActRII, ALK4, and Cripto are each indeed present in the same complexes. This finding was further confirmed by our visualization of bands corresponding to $^{125}$I-activin-A-labeled ActRII, ALK4, and Cripto following anti-FLAG immunoprecipitation (Fig. 3C, lane 3).

Having shown that activin-A can form complexes with ActRII, ALK4, and Cripto when they are overexpressed in 293T cells, we attempted to demonstrate the formation of these complexes in cells expressing these proteins at endogenous levels. Mouse embryonal carcinoma P19 cells express relatively high levels of endogenous Cripto, and we therefore subjected these cells to $^{125}$I-activin-A labeling, covalent cross-linking, and immunoprecipitation using an antibody that targets Cripto. To control for nonspecific immunoprecipitation effects of rabbit antisera, we assessed the ability of rabbit IgG to immunoprecipitate similar complexes. As shown in Fig. 3D, $^{125}$I-activin-A-labeled bands corresponding to ActRII, ALK4, and Cripto were visualized following immunoprecipitation with an anti-Cripto antibody but not with nonspecific IgG (Fig. 3D). Furthermore, the appearance of these labeled bands was almost completely abolished when cells were preincubated with 10 nM unlabeled activin-A. Significantly, this is the first evidence that demonstrates that activin-A can form receptor complexes that contain endogenous ActRII, ALK4, and Cripto.

**Nodal Competes with Activin-A for the Formation of Cripto-ActRII-ALK4 Complexes**—Our results indicate that, like Nodal, activin-A can form complexes that contain both receptor types and Cripto. Given the apparent similarity of Cripto-containing activin and Nodal complexes, we reasoned that Nodal and activin-A might compete with each other to assemble receptor complexes containing Cripto. To test this possibility, 293T cells were transfected with the indicated constructs (Fig. 4A) and then subjected to $^{125}$I-activin-A labeling in the absence or presence of 100 nM Nodal. As shown in Fig. 4A, pretreatment of cells with 100 nM Nodal substantially decreased the intensity of $^{125}$I-activin-A-labeled receptor and Cripto bands, as visualized following Cripto immunoprecipitation, indicating that Nodal competitively disrupted the formation of both activin-A-ActRII-Cripto (Fig. 4A, lanes 3 and 4) and activin-A-ActRII-ALK4-Cripto complexes (Fig. 4A, lanes 5 and 6). Interestingly, Nodal competed with $^{125}$I-activin-A cross-linking to Cripto in the absence of ALK4, further supporting previously published work demonstrating that Nodal can bind Cripto directly (12).

We predicted that activin-A and Nodal would not have additive signaling effects in the presence of Cripto, since they apparently compete to form Cripto-containing receptor complexes that elicit similar signaling responses. To test this directly, we asked whether Nodal could increase the activin-A response in the absence or presence of Cripto. 293T cells were transfected with 400 ng of vector or Cripto and then treated with 100 pM activin-A, 30 nM Nodal, or 100 pM activin-A plus 30 nM Nodal. As shown in Fig. 4B, in the absence of Cripto, activin-A elicits a ~27-fold induction of luciferase, and, as predicted, Nodal has no signaling effect either alone or together with activin-A. In cells transfected with 400 ng of the Cripto construct, the activin-A response was inhibited to ~18-fold, whereas Nodal caused an ~13-fold induction. Importantly, when these cells were treated with activin-A and Nodal together, the signaling response was essentially the same as that observed following treatment with activin-A alone (i.e. Nodal does not increase the activin-A signaling response in the presence of Cripto). These data are consistent with our cross-linking data and suggest that
Nodal and activin-A compete for access to the Cripto-bound fraction of ALK4 to form similar, low efficacy signaling complexes.

**Nodal and Activin-A Bind the Same Site on ActRII**—Next, we probed the structural similarity of Nodal and activin-A signaling complexes in more detail by testing whether these ligands bind the same ActRII ECD residues. Activin-A binds a hydrophobic surface on the ActRII ECD consisting primarily of Phe^{42}, Trp^{60}, and Phe^{83}, and mutation of any one of these three residues to alanine abolishes binding (26). Although the binding site for Nodal on ActRII has not yet been identified, it might be predicted to differ from that of activin-A, since the ActRII-binding residues on activin-A are not conserved on Nodal and since Nodal binding to ActRII requires Cripto.

To determine whether Nodal binds the same surface on the ActRII ECD as activin-A, we used transmembrane, kinase-deleted ActRII (ActRII-trunc) constructs that bind cognate ligands, form nonproductive ligand-receptor complexes, and block signaling in a dominant negative manner (28, 29). We compared the abilities of WT ActRII-trunc and the ActRII-trunc mutants F42A, W60A, and F83A to block activin-A and Nodal signaling. As a control, we also tested the function of WT ActRII-trunc and the ActRII-trunc proteins each co-precipitated with WT Cripto but not with disrupted activin-A binding (26). Importantly, the L75A, P77A, and I70A ALK4-trunc constructs did not (Fig. 6). Importantly, the L75A, P77A, and I70A ALK4-trunc. Cell lysates were then subjected to anti-FLAG immunoprecipitation followed by Western blotting using Cripto or FLAG antibodies. Alternatively, total cell extracts were subjected to Western blotting using a Cripto antibody as described under “Experimental Procedures.”

![FIGURE 5. Nodal and activin-A bind the same site on ActRII. 293T cells were transfected with A3-lux, FAST2, and CMV- or Cripto antibodies as described under “Experimental Procedures.” Cells were treated with 100 ng of empty vector or Cripto and 200 ng of ActRII-trunc constructs as indicated. Cells were treated with 100 pm activin-A or 30 nm Nodal as indicated, and resulting normalized luciferase activities are presented as -fold induction. Corresponding cell lysates were subjected to Western blotting (IB) using Myc or Cripto antibodies as described under “Experimental Procedures.”](image)

![FIGURE 6. Kinase-deleted ALK4 (ALK4-trunc) mutants with disrupted activin-A binding retain Cripto binding. 293T cells were transfected with the indicated Cripto and/or FLAG-tagged ALK4-trunc constructs with resulting cell lysates subjected to anti-FLAG immunoprecipitation (IP) followed by Western blotting using Cripto or FLAG antibodies. Alternatively, total cell extracts were subjected to Western blotting using a Cripto antibody as described under “Experimental Procedures.”](image)
sented above that activin-A and Nodal can form structurally and functionally similar receptor complexes containing Cripto.

ALK4-trunc Mutants Antagonize the Opposing Effects of Cripto on Activin-A- and Nodal-dependent Luciferase Induction—Since these ALK4-trunc mutants, deficient in activin binding, retain the ability to bind Cripto, we hypothesized that they might sequester Cripto away from ligand-receptor complexes, thereby reducing Nodal signaling on the one hand while increasing activin-A signaling on the other. To test this possibility, we asked whether these ALK4-trunc mutants could target Cripto function. 293T cells were transfected with empty vector or Cripto DNA in the absence or presence of wild-type ALK4-trunc or the indicated doses of L75A ALK4-trunc DNA together with A3-lux, FAST2, and CMV-β-galactosidase constructs as described under “Experimental Procedures.” Cells were treated with 250 pm activin-A (A) or 10 nM Nodal (B), and resulting luciferase activities were normalized relative to β-galactosidase activities and are presented as percentage of maximal induction. C, cell surface expression of Cripto and L75A ALK4-trunc was measured using an intact cell enzyme-linked immunosorbent assay as described under “Experimental Procedures.” D, Cripto and L75A ALK4-trunc constructs were transfected into 293T cells at an equimolar DNA ratio, and then resulting cell lysates were subjected to Western blotting (IB) using Cripto, FLAG, or actin antibodies as described under “Experimental Procedures.”

Activin-A and Nodal Form Analogous Receptor Complexes with Cripto via Distinct Assembly Mechanisms—In the absence of Cripto, activin-A forms high potency, high efficacy signaling complexes with ActRII and ALK4. Conversely, Nodal is incapable of signaling in the absence of Cripto (Fig. 8A). The data presented here support a model in which activin-A and Nodal each form similar signaling complexes that contain Cripto.
FIGURE 8. Criptoreducesactivin-Aefficacytolalevel similartothatofNodalandformssimilar signalingcomplexeswithbothligands. A, in the absence of Cripto, activin-A forms high potency, high efficacy signaling complexes with ActRII/IIB and ALK4, whereas Nodal does not bind these receptors or initiate signaling. B, when Cripto is present, activin-A and Nodal each form structurally similar signaling complexes that contain Cripto. Activin-A first binds to its type II receptors with high affinity and then recruits ALK4 and Cripto into a high potency, low efficacy complex. Nodal first binds Cripto before it can assemble ActRII/IIB and ALK4 into a low potency, low efficacy complex. C, diagram illustrating the ability of Cripto to modulate activin and Nodal signaling in a dose-dependent manner. In the absence of Cripto, activin signaling is high, whereas Nodal does not signal (a). As Cripto levels increase, activin signaling decreases, and Nodal signaling increases until they converge (b). D, ALK4-trunc mutants deficient in activin-A binding retain the ability to bind Cripto and block its ability to form signaling complexes with activin-A and Nodal.

(Fig. 8B). According to this model, activin-A first binds its type II receptors with high affinity and then recruits ALK4 and Cripto into high potency, low efficacy complexes. On the other hand, Nodal first binds Cripto before it can assemble ActRII/IIB and ALK4 into low potency, low efficacy complexes. Fig. 8C schematically outlines how the level of Cripto impacts Nodal and activin signaling. In the absence of Cripto (Fig. 8C, a), activin forms only high efficacy signaling complexes, whereas Nodal is unable to signal. As the level of Cripto increases, activin signaling decreases, and Nodal signaling increases until they converge (Fig. 8C, b). At this point, all receptor complexes contain Cripto and therefore have low efficacy. Finally, as shown in Fig. 8D, ALK4-trunc mutants deficient in activin-A binding retain the ability to bind Cripto and block its ability to form signaling complexes with activin-A and Nodal.

DISCUSSION

TGF-β ligands are morphogens that regulate gene expression and specify cell fate in a concentration-dependent manner (4, 31). However, Smad proteins are the central mediators of TGF-β signaling, and therefore the magnitude and duration of the Smad response are critical in determining patterns of gene expression. Importantly, Smad signaling is shaped not only by the concentration of TGF-β ligands but also by an array of cell- and ligand-specific modulators that operate through multiple mechanisms (3, 4). Cripto is one such modulator that affects the signaling of several TGF-β ligands that activate the Smad2/3 pathway. These include Nodal (13), GDF1 (32), GDF3 (33), activin-A (20), activin-B (21), and TGF-β1 (34). Interestingly, although Cripto functions as an obligatory co-receptor for Nodal, GDF1, and GDF3, it antagonizes signaling by activin-A, activin-B, and TGF-β1. Therefore, by altering its level of Cripto expression, a cell can tune its responsiveness to these ligands and vary its level of Smad2/3 signaling over a broad range.

Cripto Reduces Activin Efficacy to a Level Similar to That of Nodal—In the present study, we conducted a detailed comparison of the roles of Cripto as a modulator of activin and Nodal signaling. In contrast to previous reports exclusively postulating that Cripto inhibits activin signaling by forming inactive complexes with activin and/or its receptors, our data support a novel mechanism whereby Cripto participates in functional
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activin signaling complexes that have reduced signaling capacity. We conclude that Cripto is a noncompetitive activin antagonist, since it reduced activin efficacy without altering activin potency \( \left( \text{EC}_{50} \right) \). Also, its maximal inhibitory effect on activin signaling was partial and attainted at submaximal Cripto expression levels. In light of these findings, we propose that activin receptor complexes can exist in two signaling states, a high efficacy state in the absence of Cripto (Fig. 8, A and C, a) and a lower efficacy state in the presence of Cripto (Fig. 8, B and C, b).

According to this model, Nodal and other ligands that require Cripto can only signal in the low efficacy state. Activins, on the other hand, signal in the high efficacy state when Cripto is absent and in the low efficacy state when Cripto is present.

This two-state model of activin receptor signaling is supported by our discovery that the maximal signaling responses to activin and Nodal converge as the level of Cripto expression increases. Specifically, Cripto reduced activin-A-dependent luciferase induction and Smad2 phosphorylation to levels indistinguishable from those induced by Nodal. In each of these assays, we found that activin-A and Nodal had similar response maxima in cells expressing high levels of Cripto. However, although Cripto effectively converted activin-A into a partial agonist with efficacy resembling that of Nodal, it did not alter activin-A potency, indicating that Cripto does not affect the affinity of activin-A for ActRII/IIB. In this regard, we found that activin-A was nearly 250 times more potent than Nodal, suggesting that the affinity of activin-A for ActRII/IIB is much higher than that of Nodal for Cripto, although this remains to be tested directly. In summary, we propose that activin and Nodal form functionally similar signaling complexes in the presence of Cripto, despite the fact that these ligands have distinctly different mechanisms of receptor assembly.

Our results demonstrate that activin can elicit a substantially larger signaling response in the absence of Cripto than Nodal can generate in the presence of Cripto. This finding predicts that Nodal will be unable to regulate the expression of certain activin-responsive genes that require high levels of Smad2/3 signaling. On the other hand, our results show that activin and Nodal signaling responses converge in the presence of Cripto and therefore also predict that activin and Nodal will elicit similar biological effects on cells that express Cripto. Notably, these predictions appear to be supported by the demonstration that activin-B and Xenopus Nodal-related proteins (Xnrs) regulate the expression of disparate sets of genes during Xenopus embryogenesis, with activin-B affecting the expression of nearly twice as many genes as Xnrs (35). In addition to cooperating with Xnrs to regulate developmental genes, such as goosecoid, chordin, Xbra, Xnr2, and Derriere (36), Ramis et al. (35) showed that activin-B exclusively regulated the expression of several genes involved in cell cycle control, consistent with its antiproliferative role during gastrulation. In this regard, it is interesting to note that Smad2/3-mediated antiproliferative effects have previously been reported to require high, sustained levels of Smad2/3 signaling (37). Therefore, high efficacy activin signaling may be required to induce growth arrest during Xenopus gastrulation, whereas lower efficacy activin and Xnrs signaling in the presence of Xenopus Cripto proteins may cooperate to control mesodermal patterning. Importantly, these studies appear to be consistent with our observation that the intrinsic efficacy of activin-A is greater than that of Nodal but that these ligands signal similarly in the presence of Cripto. Further studies will be necessary to determine how Cripto expression levels shape gene transcription responses to activin and Nodal ligands.

Activin and Nodal Form Structurally Similar Complexes with ActRII, ALK4, and Cripto—The similar efficacies of activin and Nodal in the presence of Cripto suggested that these ligands assemble Cripto-containing signaling complexes that are structurally similar. In support of this, we provide biochemical evidence for complexes containing activin-A, Cripto, and both receptor types and show that the assembly of such complexes is competitively inhibited by Nodal. We further demonstrate that Nodal and activin-A share the same binding site on ActRII. This result was somewhat surprising, since Nodal, unlike activin, requires Cripto to bind type II receptors (12). Notably, activin residues that mediate type II receptor binding are not conserved in Nodal, and substitution of a 14-amino acid ActRII-binding region from activin into Nodal resulted in a chimera that could signal in a Cripto-independent manner (38). Despite these differences in their type II receptor binding properties, however, our results clearly demonstrate that activin-A and Nodal bind the same residues on ActRII. We have also shown that ALK4 has distinct and separable binding sites for activin-A and Cripto. This result supports our observation that ALK4 and Cripto are both present in complexes together with activin-A and ActRII and is also consistent with our conclusion that activin-A and Nodal form similar receptor complexes in the presence of Cripto. Together, these results indicate that despite their differing mechanisms of receptor assembly, Nodal and activin form structurally similar complexes with Cripto and activin type I and type II receptors.

Although our data indicate that Cripto is present in activin receptor complexes, they do not address the question of how the presence of Cripto in these signaling complexes reduces activin efficacy. We propose two possible models. First, although we show that Cripto does not preclude ALK4 recruitment into activin signaling complexes as was previously proposed (20), it is possible that ActRII-bound activin has lower affinity for Cripto-bound ALK4 than it has for ALK4 alone. Therefore, by reducing the affinity of activin for ALK4, Cripto could reduce the stability of activin signaling complexes and thereby reduce signaling. Such a mechanism is consistent with our observation here and previously (20) that Cripto reduces activin cross-linking to ALK4. Alternatively, Cripto may act as a wedge that distorts signaling complexes in a way that limits the ability of the ActRII kinase to phosphorylate ALK4 or the ability of ALK4 to phosphorylate Smads or both. Biochemically, these spatial constraints may explain the observed decrease in the efficiency of cross-linking between activin and ALK4 in the presence of Cripto. Such a model postulates that Cripto imposes similar structure/function constraints on signaling complexes assembled by either activin-A or Nodal and is therefore appealing, since it can explain our observation that activin-A and Nodal attain similar signaling maxima in the presence of Cripto. Three-dimensional structures of complexes containing activin or Nodal, their receptors, and Cripto will...
allow a comparison with similar structures that have been solved in the absence of Cripto (39–41) and will provide further insight into the structural basis for the effects of Cripto on signaling complexes containing ActRII/A and ALK4. According to this revised model, we predict that the three-dimensional structures of activin and Nodal in complex with Cripto and their receptors will be very similar.

**ALK4 Mutants Block Cripto Effects on Activin and Nodal Signaling**—We have shown here that three ALK4-trunc mutants each lacking activin binding were indistinguishable from WT ALK4-trunc in their ability to bind Cripto. This finding indicates that ALK4 has distinct and separable binding sites for activin and Cripto. We have further demonstrated that these ALK4-trunc mutants inhibit Cripto-dependent effects on activin and Nodal signaling, as illustrated by the L75A ALK4-trunc mutant. The effects of this mutant were dose-dependent and apparently stemmed from its ability to bind Cripto and sequester it away from ligand-receptor complexes (Fig. 8D).

However, future studies will be required in order to elucidate the precise mechanism by which these ALK4 mutants inhibit specific Cripto functions.

In addition to supporting our model in which Cripto mediates activin and Nodal signaling by forming similar signaling complexes with these ligands, the ability of these ALK4-trunc mutants to block Cripto effects on activin and Nodal signaling suggests that they may have therapeutic value as Cripto inhibitors. For example, Cripto has been identified as a marker of stem cell pluripotency (42), and it has been shown that ES cells lacking Cripto spontaneously differentiate into neurons (43–45). Therefore, developing a Cripto-blocking reagent, such as L75A ALK4-trunc, may aid efforts aimed at providing cell-based treatments for neurodegenerative disorders.

Cripto is also highly expressed in human tumors and is thought to promote tumorigenesis via multiple mechanisms (16), including antagonism of activin and Cripto. We have further demonstrated that ALK4 has distinct and separable binding sites from WT ALK4-trunc in their ability to bind Cripto. This finding the manuscript.

REFERENCES

1. Pardali, K., and Moustakas, A. (2007) *Biochim. Biophys. Acta* **1775**, 21–62
2. Massague, J., and Gomis, R. R. (2006) *FFBS Lett.* **580**, 2811–2820
3. Shi, Y., and Massague, J. (2003) *Cell* **113**, 685–700
4. Schmierer, B., and Hill, C. S. (2007) *Nat. Rev. Mol. Cell Biol.* **8**, 970–982
5. Harrison, C. A., Gray, P. C., Vale, W. W., and Robertson, D. M. (2005) *Trends Endocrinol. Metab.* **16**, 73–78
6. Piek, E., Westermark, U., Kastemar, M., Heldin, C. H., van Zoelen, E. J., Nister, M., and Ten Dijke, P. (1999) *Int. J. Cancer* **80**, 756–763
7. Mathews, L. S., and Vale, W. W. (1991) *Cell* **65**, 973–982
8. Mathews, L. S., Vale, W. W., and Kintner, C. R. (1992) *Science* **255**, 1702–1705
9. Attisano, L., Srivastava, S., and Massague, J. (1992) *Cell* **68**, 97–108
10. Carcamo, J., Wais, F. M., Ventura, F., Wieser, R., Wrana, J. L., Attisano, L., and Massague, J. (1994) *Mol. Cell. Biol.* **14**, 3810–3821
11. ten Dijke, P., Yamashita, H., Ichijo, H., Franzen, P., Lahio, M., Miyazono, K., and Heldin, C. H. (1994) *Science* **264**, 101–104
12. Yeo, C., and Whitman, M. (2001) *Mol. Cell* **7**, 949–957
13. Schier, A. F. (2003) *Annu. Rev. Cell Dev. Biol.* **19**, 589–621
14. Shen, M. (2007) *Development* **134**, 1023–1034
15. Shen, M. M., and Schier, A. F. (2000) *Trends Genet.* **16**, 303–309
16. Strizzi, L., Bianco, C., Normanno, N., and Salomon, D. (2005) *Oncogene* **24**, 5731–5741
17. Minnichetti, G., Manco, G., Parisi, S., Lago, C. T., Rosa, F., and Persico, M. G. (2001) *Development* **128**, 4501–4510
18. Yan, Y. T., Liu, J. J., Luo, E. C., Haltiwanger, R. S., Abate-Shen, C., and Shen, M. M. (2002) *Mol. Cell. Biol.* **22**, 4439–4449
19. Shen, M. M. (2003) *J. Clin. Invest.* **112**, 500–502
20. Gray, P. C., Harrison, C. A., and Vale, W. (2003) *Proc. Natl. Acad. Sci. U. S. A.* **100**, 5193–5198
21. Adkins, H. B., Bianco, C., Schiffer, S. G., Rayhorn, P., Zafari, M., Cheung, A. E., Orozco, O., Olson, D., De Luca, A., Chen, L. L., Matkowsky, K., Benjamin, C., Normanno, N., Williams, K. P., Jarpe, M., LePage, D., Salomon, D., and Sanicola, M. (2003) *J. Clin. Invest.* **112**, 575–587
22. Vaughton, J. M., and Vale, W. W. (1993) *Endocrinology* **123**, 2038–2050
23. Tsukida, K., Vaughton, J. M., Fischer, W. H., and Vale, W. W. (1995) *Endocrinology* **136**, 5493–5503
24. Harms, P. W., and Chang, C. (2003) *Genes Dev.* **17**, 2624–2629
25. Harrison, C. A., Gray, P. C., Koerber, S. C., Fischer, W., and Vale, W. (2003) *J. Biol. Chem.* **278**, 21129–21135
26. Gray, P. C., Greenwald, J., Blount, A. L., Kunitate, K. S., Donaldson, C. J., Choe, S., and Vale, W. (2000) *J. Biol. Chem.* **275**, 3206–3212
27. Topczewska, J. M., Postovit, L. M., Margaryan, N. V., Sam, A., Hess, A. R., Wheaton, W. W., Nickoloff, B. J., Topczewska, J., and Hendrix, M. J. (2006) *Nat. Med.* **12**, 925–932
28. Hemmatti-Briianlou, A., and Melton, D. A. (1992) *Nature* **359**, 609–614
29. Chen, R. H., Ebner, R., and Derynck, R. (1993) *Science* **260**, 1335–1338
30. Tsukida, K., Vaughton, J. M., Winter, P. K., and Vale, W. W. (1995) *Endocrinology* **136**, 5493–5503
31. McDowell, N., and Gorden, J. B. (1999) *Semin. Cell Dev. Biol.* **10**, 311–317
32. Cheng, S. K., Olale, F., Bennett, J. T., Briianlou, A. H., and Schier, A. F. (2003) *Genes Dev.* **17**, 31–36
33. Chen, C., Ware, S. M., Sato, A., Houston-Hawkins, D. E., Habas, R., Matzuk, M. M., Shen, M. M., and Brown, C. W. (2006) *Development* **133**, 319–329
34. Gray, P. C., Shani, G., Aung, K., Kaler, J., and Vale, W. (2006) *Mol. Cell. Biol.* **26**, 9268–9278
35. Ramis, J. M., Collart, C., and Smith, J. C. (2007) *PLoS ONE* **2**, e213
36. Piepenburg, O., Grimm, D. W., Williams, P. H., and Smith, J. C. (2004) *Development* **131**, 4977–4986
37. Nicolas, F. J., and Hill, C. S. (2003) *Oncogene* **22**, 3698–3711
38. Cheng, S. K., Olale, F., Briianlou, A. H., and Schier, A. F. (2004) *PLoS Biol.* **2**, e30
39. Thompson, T. B., Woodruff, T. K., and Jardetzky, T. S. (2003) *EMBO J.* **22**, 1555–1566
40. Greenwald, J., Vega, M. E., Allendorph, G. P., Fischer, W. H., Vale, W., and Choe, S. (2004) *Mol. Cell* **15**, 485–489
41. Allendorph, G. P., Vale, W. W., and Choe, S. (2006) *Proc. Natl. Acad. Sci. U. S. A.* **103**, 7643–7648
42. Adewumi, O., Aflatoonian, B., Ahrlund-Richter, L., Amit, M., Andrews, P. W., Beighton, G., Bello, P. A., Benvenisty, N., Berry, I. S., Bevan, S., Blum, B., Brookin, J., Chen, K. G., Choo, A. B., Churchill, G. A., Corbel,
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M., Damjanov, I., Draper, J. S., Dvorak, P., Emanuelsson, K., Fleck, R. A., Ford, A., Gertow, K., Gertsenstein, M., Gokhale, P. J., Hamilton, R. S., Hampl, A., Healy, L. E., Hovatta, O., Hyllner, J., Imreh, M. P., Itskovitz-Eldor, J., Jackson, J., Johnson, J. L., Jones, M., Kee, K., King, B. L., Knowles, B. B., Lako, M., Lebrin, F., Mallon, B. S., Manning, D., Maysah, Y., McKay, R. D., Michalska, A. E., Mikkola, M., Mileikovsky, M., Minger, S. L., Moore, H. D., Mummery, C. L., Nagy, A., Nakatsuji, N., O’Brien, C. M., Oh, S. K., Olsson, C., Otonkoski, T., Park, K. Y., Passier, R., Patel, H., Patel, M., Pedersen, R., Pera, M. F., Piekarczyk, M. S., Pera, R. A., Reubinoff, B. E., Robins, A. J., Rossant, J., Rugg-Gunn, P., Schulz, T. C., Semb, H., Sherrer, E. S., Siemen, H., Stacey, G. N., Stojkovic, M., Suemori, H., Szatkiewicz, J., Turetsky, T., Tuuri, T., van den Brink, S., Vintersten, K., Vuoristo, S., Ward, D., Weaver, T. A., Young, J. A., and Zhang, W. (2007) Nat. Biotechnol. 25, 803–816

43. Parisi, S., D’Andrea, D., Lago, C. T., Adamson, E. D., Persico, M. G., and Minchiotti, G. (2003) J. Cell Biol. 163, 303–314
44. Parish, C. L., Parisi, S., Persico, M. G., Arenas, E., and Minchiotti, G. (2005) Stem Cells 23, 471–476
45. Xu, C., Liguori, G., Adamson, E. D., and Persico, M. G. (1998) Dev. Biol. 196, 237–247