Nodal Signaling Uses Activin and Transforming Growth Factor-β Receptor-regulated Smads*

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Nodal, a member of the transforming growth factor β (TGF-β) superfamily, is implicated in many events critical to the early vertebrate embryo, including mesoderm formation, anterior patterning, and left-right axis specification. Here we define the intracellular signaling pathway induced by recombinant nodal protein treatment of P19 embryonal carcinoma cells. Nodal signaling activates pAR3-Lux, a luciferase reporter previously shown to respond specifically to activin and TGF-β. However, nodal is unable to induce pTlx2-Lux, a reporter specifically responsive to bone morphogenetic proteins. We also demonstrate that nodal induces p(CAGA)12, a reporter previously shown to be specifically activated by Smad3. Expression of a dominant negative Smad2 significantly reduces the level of luciferase reporter activity induced by nodal treatment. Finally, we show that nodal signaling rapidly leads to the phosphorylation of Smad2. These results provide the first direct biochemical evidence that nodal signaling is mediated by both activin-TGF-β pathway Smads, Smad2 and Smad3. We also show here that the extracellular cripto protein is required for nodal signaling, making it distinct from activin or TGF-β signaling.

Members of the transforming growth factor β (TGF-β)1 superfamily of intercellular signaling factors regulate cell fate and behavior during development and in the adult (1). The three major subgroups based on sequence similarity are the TGF-βs, activins and inhibins, and bone morphogenetic proteins (BMPs; Ref. 1). Nodal and related factors form a separate subgroup and are implicated in many events critical to the early vertebrate embryo, including mesoderm formation, anterior patterning, and left-right axis specification (2).

Signaling by TGF-β and related ligands uses two types of receptors, type I and type II transmembrane serine-threonine kinases. Ligand binding results in the formation of heteromeric receptor complexes, in which type II receptors phosphorylate type I receptors (1, 3). Downstream signal transduction events are mediated by the intracellular Smad proteins. One class, the receptor-regulated Smads (R-Smads), are directly phosphorylated by activated type I receptors on a C-terminal SSXS motif. Upon phosphorylation, R-Smads form complexes with the co-Smad, Smad4 and then translocate to the nucleus and regulate transcription of target genes. Biochemical and biological studies have established that the R-Smads used by TGF-β and activin signaling, Smad2 and Smad3, are distinct from those used by BMP signaling, Smad1, Smad5, and Smad8 (1, 3, 4).

The nodal signaling pathway awaits characterization at the biochemical level. However, mutational studies in the mouse and zebrafish and ectopic expression studies in Xenopus and zebrafish suggest that the nodal and activin signaling pathways may share receptors and Smads. Targeted mutations in the mouse Smad2 gene (5–8) and the activin type IB receptor gene (9) and combined mutations of the activin type IIA and IIB receptor genes (10) show gastrulation phenotypes resembling the mouse nodal mutant (11, 12). Nodal and activin have similar mesoderm-inducing capacity in Xenopus embryo explants or in whole embryos (13), whereas dominant negative forms of Smad2 (14) or activin receptors (15–17) disrupt endogenous mesoderm formation. One difference between nodal and activin signaling has been described in zebrafish. There is genetic evidence that the extracellular epidermal growth factor-Cripto, FRL1, Cryptic (EGF-CFC) protein one-eyed pinhead is required for signaling by zebrafish nodal-related factors but not for activin signaling (18). Whether nodal signaling in the mouse also depends on the function of the related EGF-CFC proteins crypt (19) and cryptic (20) has not been established. However, targeted mutations in both genes result in embryonic lethal phenotypes that are suggestive; cryptic null embryos exhibit gastrulation defects (21, 22), and the absence of cryptic leads to left-right defects (23, 24).

Earlier studies on nodal function relied on overexpression from transfected or injected expression vectors. Here we describe the production of recombinant nodal protein. The availability of purified nodal protein allows functional characterization of this developmentally important pathway not previously possible. We describe a quantitative assessment of nodal signaling activity in P19 mouse embryonal carcinoma cells, a model system for the early mouse embryo (25). We have determined that P19 cells are competent to respond to nodal signaling and have exploited this capacity and the availability of recombinant nodal protein to investigate the intracellular pathway by which the nodal signal is transduced. In this study we provide direct evidence that nodal signals through activin-TGF-β pathway-specific Smads but not BMP pathway-specific...
Smads. In addition, we show that nodal activity depends on the expression of cripto, suggesting a general requirement for EGF-CFC function in vertebrate nodal signaling.

**EXPERIMENTAL PROCEDURES**

**Recombinant Nodal Protein**—The nature region of human nodal protein was expressed in *Escherichia coli* and refolded essentially as described (26). Nodal dimer was isolated by ion exchange chromatography on S-Sepharose Fast Flow (Amersham Pharmacia Biotech) using a gradient from 0.05 to 1.0 M NaCl in 25 mM NaAc, 30% isopropanol (pH 4.0), followed by reversed phase high-performance liquid chromatography over a 2.0 × 0.46-cm C4 column (Supelco, Bellefonte, PA) on an acetonitrile gradient in 0.1% trifluoroacetic acid. Protein purity was assessed by SDS-polyacrylamide gel electrophoresis, and concentration was determined by amino acid analysis. Protein activity was assessed by measuring the luciferase activity was measured 60 min after measuring luciferase activity. All assays were performed in duplicate. Luciferase activity was normalized to β-galactosidase activity.

**Immunoblot Analysis**—P19 cells were plated in six-well plates (2 × 10^5 cells/well) in 2 ml of media on day 0. On day 2, media were removed and replaced with DMEM containing 0.1% bovine serum albumin for 3 h. Nodal, activin, or BMP was then added in 0.1% bovine serum albumin for varying times. Cells were washed with ice-cold phosphate-buffered saline, and 200 μl of 1× gel-loading dye was added to each well. Cell lysates were collected, sonicated for 15 s, and heated for 3 min at 95 °C. Samples were fractionated by SDS-polyacrylamide gel electrophoresis using a 10–20% gradient acrylamide gel. Transfer to the polyvinylidene difluoride membrane and processing were as described previously (34, 35). Briefly, membranes were incubated in TNT 20 (20 mM Tris-HCl (pH 7.6), 137 mM NaCl, 0.1% w/v Tween 20) with 5% w/v nonfat dried milk for 1 h, washed in TNT 20 (3–5 min), and incubated with primary antibody (1:1000) in TNT 20 containing 1% milk. This was done for 1 h at room temperature for non-phospho-specific antibodies and overnight at 4 °C for phospho-specific antibodies. After further washing in TNT 20, membranes were incubated for 1 h with horseradish peroxidase-linked anti-IgG secondary antibody (Pierce; 1:5000), and immunoreactive proteins were detected using SuperSignal chemiluminescent substrate (Pierce).

**RESULTS**

**P19 Embryonal Carcinoma Cells Are Responsive to Recombinant Nodal Protein**—Unpublished work from our laboratory has shown that P19 embryonal carcinoma cells stably transfected with a nodal expression vector can differentiate into mesoderm without chemical induction. 2 These results suggested that P19 cells are responsive to nodal and may provide an appropriate model system to study nodal function in the early mouse embryo. To confirm these results and establish a reporter assay to dissect the nodal signaling pathway, we carried out transient transfection of undifferentiated P19 cells using the p(SBE)4 luciferase reporter. p(SBE)4 has been shown to be a general reporter for the TGF-β superfamily in HepG2 cells, responding to TGF-β itself, activin, and BMP2 (30). Therefore, it was a good candidate reporter to confirm nodal signaling in P19 cells without knowing any details of the pathway. We first determined that p(SBE)4 is induced by TGF-β, activin, and BMP2 in P19 cells. As shown in Fig. 1A, activin (20 ng/ml), BMP4 (40 ng/ml), and TGF-β1 (1 ng/ml) increased p(SBE)4 reporter activity by 5 ± 1.8, 4 ± 2.3, and 3 ± 0.4-fold (mean ± S.D.), respectively. These results established that p(SBE)4 is a general reporter for both TGF-β-activin and BMP pathway-specific R-Smad activation in P19 cells, as in HepG2 cells.

To analyze nodal signaling, we used recombinant human nodal protein, produced in bacteria and refolded in vitro. Nodal protein activity was assayed using *Xenopus* embryo explants. Animal caps treated with nodal protein (at 5 μg/ml) or activin (at 2.5 or 25 ng/ml) underwent elongations characteristic of mesoderm formation (data not shown), confirming the bioactivity of the recombinant protein preparation. We then evaluated p(SBE)4 activation by recombinant nodal treatment of P19 cells. As shown in Fig. 1B, nodal induced p(SBE)4 reporter activity in a dose-dependent manner with a 14-fold increase at 4 μg/ml. These studies confirmed that P19 cells are competent to respond to nodal and provide an experimental system for defining the intracellular signaling pathway. In further studies, we used 2 μg/ml nodal, because this amount still gave a reporter gene assay kit (Tropi; Applied Biosystems, Foster City, CA) as specified by the manufacturer, in an EG&G Berthold Lumat LB 9507 luminometer. Briefly, cells were washed once with phosphate-buffered saline. After addition of 120 μl of lysis buffer, cells were scraped and centrifuged (4 °C, 12,000 × g, 5 min). 10 μl of the supernatant was added to 50 μl of buffer A followed by 100 μl of buffer B. After a delay of 2 s, the luciferase signal was measured for 5 s. β-Galactosidase activity was measured 60 min after measuring luciferase activity. All assays were performed in duplicate. Luciferase activity was normalized to β-galactosidase activity.

**Luciferase and β-Galactosidase Activity Measurements**—Luciferase and β-galactosidase activity were measured using the Dual light chemiluminescent reporter gene assay kit (Tropi; Applied Biosystems, Foster City, CA) as specified by the manufacturer, in an EG&G Berthold Lumat LB 9507 luminometer. Briefly, cells were washed once with phosphate-buffered saline. After addition of 120 μl of lysis buffer, cells were scraped and centrifuged (4 °C, 12,000 × g, 5 min). 10 μl of the supernatant was added to 50 μl of buffer A followed by 100 μl of buffer B. After a delay of 2 s, the luciferase signal was measured for 5 s. β-Galactosidase activity was measured 60 min after measuring luciferase activity. All assays were performed in duplicate. Luciferase activity was normalized to β-galactosidase activity.

**Animals**—Xenopus laevis embryos were obtained from Harlan Sprague-Dawley (Indianapolis, IN) and were held at 20 °C until used on day 0. On day 2, animals were removed and replaced with media containing 50% bovine serum albumin for 3 h. Nodal, activin, or BMP was then added in 0.1% bovine serum albumin for varying times. Cells were washed with ice-cold phosphate-buffered saline, and 200 μl of 1× gel-loading dye was added to each well. Cell lysates were collected, sonicated for 15 s, and heated for 3 min at 95 °C. Samples were fractionated by SDS-polyacrylamide gel electrophoresis using a 10–20% gradient acrylamide gel. Transfer to the polyvinylidene difluoride membrane and processing were as described previously (34, 35). Briefly, membranes were incubated in TNT 20 (20 mM Tris-HCl (pH 7.6), 137 mM NaCl, 0.1% w/v Tween 20) with 5% w/v nonfat dried milk for 1 h, washed in TNT 20 (3–5 min), and incubated with primary antibody (1:1000) in TNT 20 containing 1% milk. This was done for 1 h at room temperature for non-phospho-specific antibodies and overnight at 4 °C for phospho-specific antibodies. After further washing in TNT 20, membranes were incubated for 1 h with horseradish peroxidase-linked anti-IgG secondary antibody (Pierce; 1:5000), and immunoreactive proteins were detected using SuperSignal chemiluminescent substrate (Pierce).

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significant level of induction within the range of activation induced by the other ligands.

Nodal Signaling Requires EGF-CFC Function—The specific activity of recombinant nodal protein was low compared with the other ligands tested, perhaps because of a significant amount of inactive protein in the high-performance liquid chromatography-purified fraction. The requirement of using such large amounts of nodal protein raised the question of specificity: is the signaling truly representative of endogenous nodal signaling, or is it caused by low-affinity binding to nonphysiological receptors? To address this question, we analyzed nodal signaling in HepG2 cells. As shown in Fig. 2A, activin, BMP4, and TGF-β(1 ng/ml) induced p(SBE)₄ activity in transfected HepG2 cells. The mean + S.D. for six experiments is shown. B, treatment of P19 cells with increasing concentrations of nodal (0.25–4 μg/ml) leads to an increase in p(SBE)₄ activity. The mean + S.D. of two experiments is plotted.

expression vector could rescue nodal signaling in HepG2 cells. As shown in Fig. 2B, nodal treatment of HepG2 cells expressing cripto led to a significant increase in p(SBE)₄ activity (4.92 ± 0.95-fold) compared with controls. This result provides further support that the recombinant protein is acting physiologically and suggests that EGF-CFC activity is a general requirement for vertebrate nodal signaling.

Nodal Induces a TGF-β and Activin Response but Not a BMP Response—To determine whether intracellular nodal signals are mediated through the TGF-β-activin or BMP pathway, or both, we used two reporters that differentiate between these pathways. pAR3-lux contains an activin response element from the Xenopus Mix2 gene and is activin and TGF-β inducible in Xenopus embryos (36) and in HepG2 cells (32). The pTlx2-lux reporter contains a BMP-responsive element and is BMP2, BMP7, and Smad1 inducible in P19 cells (33, 37). pTlx2-lux has also been shown to be induced by constitutively active forms of the BMP-specific type I receptors activin receptor-like kinase 2 and activin receptor-like kinase 6 but not by a constitutively active activin receptor-like kinase 4 receptor, an activin-specific type I receptor (37). We transiently transfected P19 cells with pAR3-lux and pTlx2-lux reporters and treated them with nodal (2 μg/ml), activin (20 ng/ml), BMP4 (40 ng/ml), and BMP6 (40 ng/ml). As shown in Fig. 3A, activin treatment of cells transiently transfected with pAR3-lux resulted in a 4.8 ± 0.9-fold induction of luciferase activity, consistent with previous reports (37). When cells were treated with nodal, pAR3-lux activation increased by 3 ± 0.4-fold. Neither BMP4 nor BMP6

3 D. Salomon, personal communication.
Luciferase activity in the absence of any factor is taken as 100%.

**a** To determine the involvement of Smad2 in nodal signaling, we first used a dominant negative approach. Previous work has shown that C-terminal serines in Smad2 are phosphorylated by the TGF-β receptor, and mutating these serines to alanines prevents Smad2 phosphorylation and its nuclear accumulation (41). These mutant forms of Smad2 can still associate with the receptor, however, and can act as dominant negatives. We used the dominant negative Smad2 (3S-A) to test whether it affected nodal-mediated p(SBE)4 activation. P19 cells were transfected with p(SBE)4 either alone or together with Smad2 (3S-A), and luciferase activity was measured after treatment with nodal or activin. The results are shown in Fig. 5A. In the absence of Smad2 (3S-A), p(SBE)4 activity was increased 6- and 9-fold by nodal and activin, respectively, which was taken as 100% activity. In the presence of Smad2 (3S-A), we observed only 3.96- and 6.75-fold induction by nodal and activin, respectively, a 34 and 25% reduction. We repeated these experiments using the activin- and TGF-β-specific reporter pAR3-lux. As shown in Fig. 5B, expression of the dominant negative Smad2 led to a 40% reduction of nodal-induced pAR3-lux activity. These results indicate that nodal also can use Smad2 for intracellular signaling in P19 cells.

**b** Nodal Signaling Results in Phosphorylation of Smad2—As a second approach to determine Smad2 involvement in nodal signaling, we examined Smad2 phosphorylation after nodal treatment of P19 cells. These studies used antisera specific for the C-terminal phosphorylated form of Smad2. These antibodies do not recognize the unphosphorylated (nonactivated) forms of these Smads. P19 cells were treated with nodal (2 μg/ml), activin (20 ng/ml), or BMP4 (40 ng/ml) for various times, and cell lysates were analyzed by Western blotting. As shown in Fig. 6A, nodal activated Smad2 within 15 min, and phosphorylation was found for at least 4 h before returning to basal levels by 24 h. Activin treatment also led to Smad2 phosphorylation in P19 cells. However, peak levels for activin stimulation were seen at 1 h with a return to basal level by 4 h.

To confirm that nodal signaling does not go through BMP R-Smads, we used antisera specific for the C-terminal phosphorylated forms of Smads 1, 5, and 8. As shown in Fig. 6B, BMP4 treatment led to rapid phosphorylation of these BMP R-Smads, which returned to basal levels by 24 h. However, treatment with nodal had no effect. These results provide direct evidence that nodal signals through Smad2 and conclusively show that BMP R-Smads are not used.

**DISCUSSION**

We have obtained several lines of evidence indicating that nodal activity in P19 cells is regulated through the activin-TGF-β pathway mediated by Smad2 and Smad3 and not through BMP-regulated Smads. First, nodal induces pAR3-lux, which was previously shown to be specifically responsive to TGF-β and activin (37). Nodal does not activate pTlx2-lux, which has been shown to be strictly BMP inducible (33, 37). Second, nodal activates the p(CAGA)12 reporter, which was previously shown to be activated specifically by Smad3 (31). Third, nodal activation of either p(SBE)4 or pAR3-lux is re-
duced significantly by expression of a dominant negative form of Smad2. Fourth, nodal treatment of P19 cells rapidly induces the phosphorylation of Smad2 but not BMP R-Smads.

The involvement of Smad2 in nodal signaling in P19 cells suggests that a similar pathway exists in the intact embryo. Several different targeted mutations of Smad2 in the mouse germ line have been reported (5–8). The studies by Nomura and Li (7) and Weinstein et al. (6) have described gastrulation defects similar to those of the nodal null mutant, which lacks mesoderm formation (11, 12, 42). The similarity of these phenotypes is consistent with nodal signaling using Smad2 in the gastrulating embryo. Nomura and Li (7) also reported that embryos heterozygous for both the Smad2 and nodal mutations have phenotypic defects in left-right axis development and anterior patterning. Heyer et al. (8) reported similar phenotypes in Smad2 mutant embryos rescued through gastrulation. These latter phenotypes are similar to that produced by a hypomorphic nodal mutation, indicating that nodal signaling uses Smad2 in developmental processes other than mesoderm formation. However, the Smad2 knockouts reported by Waldrip et al. (5) and Heyer et al. (8) showed transient mesoderm formation, complicating the conclusion that nodal uses Smad2 in the induction of mesoderm in the embryo. Our conclusive demonstration that nodal signals through Smad2 in P19 cells will aid in the interpretation of these different Smad2 knockout phenotypes.

We have shown that nodal signaling also can use Smad3 in P19 cells. However, targeted mutation of Smad3 in the mouse does not lead to early developmental defects (43–45), suggesting that Smad3 is not involved in nodal signaling in the embryo or that Smad2 can compensate for the loss of Smad3. Interestingly, embryos heterozygous for both Smad2 and Smad3 can display left-right abnormalities. This finding suggests that nodal signaling may indeed involve both Smad2 and Smad3 in the embryo.

Although we show here that nodal uses an activin-like intracellular signaling pathway, we have also found some major differences between nodal and activin signaling. We have found that human HepG2 cells are not responsive to human nodal protein unless transfected with an expression vector encoding the extracellular EGF-CFC protein cripto. Previous genetic studies demonstrated a requirement for the zebrafish EGF-CFC protein one-eyed pinhead in nodal-related signaling in the zebrafish but not for activin signaling (18). A recent study showed that injection of a mouse nodal expression vector into Xenopus embryos resulted in induction of a luciferase reporter carrying an enhancer element from the nodal gene, but at a significantly lower level than that obtained with an activin expression vector. However, co-injection of nodal and cripto expression vectors led to similarly high levels as with the activin expression vector (46). Together these data suggest that there is a general requirement for EGF-CFC protein function in vertebrate nodal signaling.

Another difference between activin and nodal signaling is in demonstration that nodal signals through Smad2 in P19 cells will aid in the interpretation of these different Smad2 knockout phenotypes.

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4 L. A. Lowe, S. Yamada, and M. R. Kuehn, submitted for publication.

5 M. Weinstein and C. X. Deng, personal communication.
the persistence of Smad2 phosphorylation occurring in response to these two factors. We found that during continuous activin treatment of P19 cells the levels of activated Smad2 peaked at 1 h and then declined. This is similar to what was found for Smad2 activated by continuous TGF-β treatment of HaCaT cells (47). However, Smad2 phosphorylation induced by continuous nodal treatment of P19 cells was sustained for much longer. This difference may be attributable to differences at the level of receptor-mediated phosphorylation of Smad2 or the level of Smad2 at the level of receptor-mediated phosphorylation of Smad2 or activin. Alternatively, the receptor complex brought together by nodal may be more stable than that formed by TGF-β or activin receptor binding, perhaps through the action of extracellular EGF-CFC proteins. Increased receptor complex stability might result in binding, perhaps through the action of extracellular EGF-CFC proteins. Increased receptor complex stability might result in more sustained activation of Smad2.

In conclusion, we have shown that nodal signaling in P19 cells is mediated intracellularly through the TGF-β-activin pathway, providing important biochemical evidence that nodal signaling in the embryo uses Smad2 and Smad3.

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