Cited1 Is a Bifunctional Transcriptional Cofactor That Regulates Early Nephronic Patterning

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In a screen to identify factors that regulate the conversion of mesenchyme to epithelium during the early stages of nephrogenesis, it was found that the Smad4-interacting transcriptional cofactor, Cited1, is expressed in the condensed cap mesenchyme surrounding the tip of the ureteric bud (UB), is downregulated after differentiation into epithelia, and has the capacity to block UB branching and epithelial morphogenesis in cultured metanephroi. Cited1 represses Wnt/β-catenin but activates Smad4-dependent transcription involved in TGF-β and Bmp signaling. By modifying these pathways, Cited1 may coordinate cellular differentiation and survival signals that regulate nephronic patterning in the metanephros.

Epidelial morphogenesis in the developing kidney is regulated by reciprocal interactions between ureteric bud (UB) epithelia and the metanephric mesenchyme (MM). During the early stages of renal development, the UB invades the overlying intermediate mesoderm and induces a subset of mesenchymal cells to undergo condensation before the development of polarized nephronic epithelia (1). The initial condensate covers the tips of the UB and is referred to as the cap condensate. Most of these cells are relatively quiescent, but a small number at the lateral edge of the cap undergo a burst of proliferation associated with the formation of pretubular aggregates that express a distinct profile of genes, including Wnt4, Lim1, and Sfrp2 (2,3). Detailed morphometric analysis suggests that subsequent differentiation and patterning of these pretubular aggregates into tubular and glomerular epithelia occurs through a temporally distinct set of inductive signals (4).

Although the signaling pathways regulating the later stages of nephronic patterning are poorly understood, genetic studies in mice have identified distinct signaling events that are required for the formation of the cap condensates and pretubular aggregates. For example, the secreted TGF-β superfamily growth factor, Bmp7, plays a critical role in recruitment and survival of the cap mesenchyme (5,6), whereas Wnt4 is required for the formation of pretubular aggregates in vivo (7). However, it is unclear whether additional regulatory mechanisms are required to coordinate these signaling pathways during the early phases of nephronic induction.

In a screen to identify factors involved in regulating these early responses, we now demonstrate that Cited1 is expressed in the cap mesenchyme and is downregulated as these cells form pretubular aggregates and differentiate into early nephronic epithelia. Formerly known as Msg1, Cited1 is the founding member of a family of transcriptional cofactors and has been identified in several embryonic tissues (8) and malignancies (9–11). Four members of the Cited family of proteins have been identified (12–17): Cited1/Msg1, Cited2/Mrg1, Cited3 (Xenopus, zebrafish, red carp, and chicken—no mammalian orthologues identified), and Cited4/Mrg2. These proteins have approximately 200 amino acid residues and share a conserved C-terminal transcription activation domain, the CR2 domain (14). All of the Cited proteins bind directly to the transcriptional integrators CBP and p300 through conserved “LPXL” sequence motifs in their CR2 domains (18), and this interaction is required for their transactivating activities. For example, Cited1 activates TGF-β and estrogen-dependent transcription mediated by Smad4 and estrogen receptors, respectively, and these effects are dependent on binding of the CR2 domain of Cited1 to CBP and p300 (12,19,20). On this basis, Cited1 is thought to target specific transcriptional responses through interactions with other transcription factors and to modify these responses through interaction with the CBP and p300 co-activators.
In this article, we show that overexpression of Cited1 blocks UB branching and epithelial morphogenesis in cultured embryonic kidneys and acts as a bifunctional regulator of transcription, activating TGF-β family signals while repressing Wnt/β-catenin–dependent responses. Because these signaling pathways play essential roles in the regulation of nephronic patterning, these findings suggest that the coordinated regulation of Cited1 in the cap condensate may play a critical role in regulating cellular responses during renal development.

Materials and Methods

Mesenchyme Cultures

Whole kidneys and isolated preparations of MM dissected away from T-shaped UB structures were prepared from E13.5-dpc rat embryos and cultured as described previously (3, 26). Cultured mesenchymes were treated with a 50% stock solution of conditioned media from a rat UB cell line (RUBI-CM) for 72 h to induce epithelial differentiation, as described previously (26). Explants were assessed by immunofluorescence staining for epithelial structures, whereas morphology of the induced mesenchyme cultures was assessed by photomicroscopy.

Protein Transduction

Rat Cited1 cDNA was subcloned into pTAT-HA vector, kindly provided by Steve Dowdy, which includes a polyhistidine tag upstream of the TAT sequence to enable purification of the recombinant protein (24). The recombinant TAT fusion proteins were expressed in BL21-AI cells, expression induced with arabinose for 4 hours at 37°C, lysed, and sonicated in denaturing buffer containing 6 mol guanidine hydrochloride, 500 mmol NaCl, and 20 mmol NaHPO₄ pH 7.4. After spinning down the bacterial pellet, the supernatant was incubated with nickel sepharose resin (ProBond, Invitrogen) overnight at 4°C before washing extensively in 8 mol urea, 500 mmol NaCl, and 20 mmol NaHPO₄. Initial washes were performed with this buffer adjusted to pH 7.4, followed by pH 6.0 and pH 5.5, and finally eluting the His-tagged TAT fusion protein in the same buffer at pH 4.0. Aliquots from individual elution fractions were separated by SDS-PAGE and purity evaluated by immunoblotting with anti-His antibodies. The His-tagged TAT fusion protein solution was mixed with the organ culture media and transfected into cultured kidneys, activating TGF-β family signals while repressing Wnt/β-catenin–dependent responses. Because these signaling pathways play essential roles in the regulation of nephronic patterning, these findings suggest that the coordinated regulation of Cited1 in the cap condensate may play a critical role in regulating cellular responses during renal development.

Xenopus Embryo Manipulation and Microinjection

Xenopus embryos were obtained by in vitro fertilization (48), maintained in 10% normal amphibian medium (NAM) (49), and staged according to Nieuwkoop and Faber (50). Synthetic capped RNA was made from linear pCS2-Cited1 and injected into the embryos as indicated in the figure legends. The average dorsal anterior index was determined to quantify the degree of loss of axial structures, as described previously (40).

Expression Plasmids

Mammalian Cited1, β-catenin, p300, Tcf, and Lef expression plasmids and the luciferase reporters have previously been described (12, 29, 31, 35, 51–53) and were gifts from Stephen Byers, Wiebe Kruijer, Randall Moon, Vasily Ozyroko, Harold Varmus, Bert Vogelstein, and Jeff Wrana. Selected β-catenin and Cited1 constructs were subcloned into pCS2 for Xenopus expression studies, and GST–β-catenin was provided by David Rim. The TAT–Cited1 fusion protein was generated by subcloning full-length rat Cited1 cDNA in frame into the bacterial expression vector pTAT (a gift from Steve Dowdy) (24).

Transfection and Transcriptional Activation Assay

Cells were transiently transfected using LipofectAMINE (Invitrogen), and transfection efficiency was evaluated by cotransfecting the β-galactosidase expression plasmid pSV–β-galactosidase and by enzyme activity assay. For the β-catenin transcription assays, HepG2 cells were transfected with HA-tagged Cited1 or its deletion mutants, V5-tagged Tcf4 or Lef1, and the OT-Lux reporter containing three Lef1/Tcf consensus binding sites upstream of a minimal promoter (35). For the Gal4 transcription assays, HepG2 cells were transfected with HA-tagged Cited1 or its deletion mutants, V5-tagged Tcf4 or Lef1, and the OT-Lux reporter containing three Lef1/Tcf consensus binding sites upstream of a minimal promoter (35). For the Gal4 transcription assays, HepG2 cells were transfected with Gal4–β-cateninR10C, encompassing the C-terminal 520 to 781 residues of Xenopus β-catenin fused to the Gal4 DNA-binding domain (31), and the Gal4 pG5–E1B-Lux reporter (52). Cells were lysed after 42 h, and luciferase and β-galactosidase activity was determined, as described previously (54). Luciferase values were corrected for transfection efficiency with β-galactosidase and expressed as the mean ± SEM of triplicate measurements.
Immunoprecipitation, Western Blots, and GST Pull-Down Assays

293 cells were lysed in buffer containing 1% TritonX-100, 100 mmol NaCl, 25 mmol Hepes pH 7.5, 5 mmol EDTA, and 10% glycerol in the presence of phosphatase and protease inhibitors. Lysates were either directly separated by SDS-PAGE and transferred to Immobilon-P membranes (Millipore) and/or first immunoprecipitated using specific rabbit antibodies raised against the C-terminal domain of β-catenin (Sigma), the Cited1 SID (E62319M) (14,42), or control rabbit IgG. Immunoprecipitates were subjected to Western blot after SDS-PAGE using the corresponding β-catenin (Transduction Laboratories) or Cited1 mouse monoclonal antibody, 2H6, raised against full-length GST–Cited1 fusion protein.

For in vitro pull-down assays, GST or GST–β-catenin were expressed in bacteria and purified using glutathione agarose beads, according to the manufacturer’s protocol (Amersham). These were then incubated with [35S]-methionine–labeled, in vitro–transcribed, and translated Cited1 or p300 fragment protein products, made using the rabbit reticulocyte lysate T7 coupled transcription/translation system from Promega. Mixtures were incubated for 2 hours in GST-binding buffer (0.05% NP-40, 75 mmol KCl, 20 mmol Hepes pH 7.5, 0.5 mmol EDTA, 5 mmol MgCl2, 2 mmol DTT, 0.02% BSA, and 20% glycerol), and washed five times in GST wash buffer (0.5% NP-40, 150 mmol NaCl, 20 mmol Tris pH 8, and 1 mmol EDTA). GST fusion protein complexes were eluted from the glutathione beads, separated by SDS-PAGE, and the [35S]-labeled interacting components were identified by autoradiography.

Results
Cited1 Is Expressed in the Cap Condensate

We used differential display to identify genes regulated in cultured rat MMs after induction of epithelial morphogenesis by RUB1-CM (21). One of these was the transcriptional cofactor Cited1. To determine the significance of this response, we mapped the expression of Cited1 in developing rat and mouse kidneys. Cited1 mRNA and protein were detected in the condensed mesenchyme within the outer nephrogenic zone of the developing kidneys, restricted to the cap condensate surrounding UB tips in both rat and mouse kidneys (Figure 1). Higher-magnification images demonstrate that it is no longer detectable in pretubular aggregates at the lateral edge of the condensate (Figure 1, B and F, arrows). These findings indicate that Cited1 has a highly restricted expression pattern in the metanephric kidney and suggest that it may play a role in regulating the functional properties of cells within the cap condensate.

Given its restricted pattern of expression, we explored the regulation of Cited1 by inductive signaling during the early phases of metanephric differentiation. Cited1 mRNA and protein were expressed in uninduced rat E13.5 cap mesenchyme and were downregulated in these explant cultures after 24 and 72 h of treatment with the RUB1-CM (Figure 2). Semiquantitative reverse-transcription PCR demonstrated reciprocal changes in the expression of Cited1 and the epithelial markers Lim1 and Sfrp-2 (Figure 2A), whereas immunohistochemical analysis of induced explants showed loss of expression of Cited1 in tubular epithelial structures after 72 h of treatment with inductive media (Figure 2C, white arrows). Furthermore, as described previously (3), addition of the secreted Wnt antagonist, Sfrp1, resulted in the expansion of the undifferentiated mesenchyme after induction and was associated with persistent expression of Cited1 (Figure 2D). These findings indicate that

Figure 1. Cited1 expression in the developing kidney. E19 (A to C) and E16 (D) rat and E16 mouse (E, F) embryonic kidneys were fixed and the expression of Cited1 protein was analyzed by immunohistochemistry using J72220K anti-Cited1 antibody (A, B, D to F) or in situ hybridization with rat anti-sense Cited1 (C). (B, F) Areas of high magnification from the rat E19 and mouse E16 kidneys, respectively. Arrows indicate transition between Cited1 staining MM and unstained epithelia within the nephrogenic vesicles.
Cited1 expression is closely linked to epithelial differentiation in the MM.

**Cited1 Blocks UB Branching and Epithelial Morphogenesis in Metanephric Organ Culture**

To explore the functional effects of Cited1 during nephrogenesis, we analyzed the effect of overexpressing Cited1 on branching and epithelial differentiation in cultured rat metanephiroi. Because our early attempts to overexpress Cited1 using various DNA transfection reagents, electroporation, and adenoviral and retroviral delivery systems failed to achieve efficient tissue penetration, we used a modified protein transduction system that has been used to overexpress a number of functionally active intracellular proteins in a variety of cells and tissues (22–24). For this, we first generated a TAT-Cited1 fusion protein that incorporates the membrane transduction HIV TAT peptide motif and a polyhistidine tag to facilitate purification. Two technical issues that had to be addressed were low yields of bacterially expressed TAT–Cited1 fusion protein using isopropylthiogalactopyranoside (IPTG)-inducible bacterial expression systems and poor solubility of the purified recombinant protein after removal of urea buffer that is required to maintain the recombinant proteins in a denatured form during the purification procedure (24). These were addressed by switching to more tightly controlled, arabinose-inducible BL21-AI *Escherichia coli* cells (Invitrogen) for protein synthesis, and by avoiding the use of desalting columns to remove urea by ethanol precipitation and washing of the protein preparations. The precipitated protein was then resuspended in bicarbonate buffer, and the freshly prepared TAT fusion protein was applied directly to the kidney explants. To determine the efficiency and penetration of TAT–Cited1 in metanephric organ cultures, purified FITC-conjugated TAT and TAT–Cited1 were incubated with E13.5 rat MM. (B to D) Expression of Cited1 in differentiating rat MM. Isolated mesenchymes were cultured for 72 h with Fgf2 (B), Fgf2 with RUB1-CM (C), or Fgf2 with RUB1-CM and the soluble Wnt antagonist Sfrp1 (D). Cited1 was detected using affinity purified rabbit anti-Cited1 antibody, J7220K. White arrows show Cited1-negative tubular epithelial structures in the induced mesenchyme. Polycarbonate filters appear as white bands on the images.

Figure 2. Regulation of Cited1 expression in cultured MM. (A) Reverse-transcription PCR for Cited1 expression in isolated rat metanephric mesenchymes (MM). RNA from isolated rat MMs cultured with Fgf2 (white bars) or Fgf2 and conditioned media from a rat UB cell line (RUB1-CM) (black bars) for 24 and 72 h was amplified by reverse-transcription PCR. Expression of Cited1 and the early epithelial cell markers *Lim1* and *Sfrp2* were quantified by densitometry of ethidium bromide–stained gels, and results were expressed as the ratio of specific mRNA to *Gapdh* intensities. Results are representative of two replicate experiments of pooled mesenchymes from multiple litters. Each individual point was derived from 12 isolated, pooled, and cultured E13.5 rat MM. (B to D) Expression of Cited1 in differentiating rat MM. Isolated mesenchymes were cultured for 72 h with Fgf2 (B), Fgf2 with RUB1-CM (C), or Fgf2 with RUB1-CM and the soluble Wnt antagonist Sfrp1 (D). Cited1 was detected using affinity purified rabbit anti-Cited1 antibody, J7220K. White arrows show Cited1-negative tubular epithelial structures in the induced mesenchyme. Polycarbonate filters appear as white bands on the images.
from day 13.5 postcoitum rat embryos and cultured as described previously (3). Untreated explants demonstrated typical branching of the UB (yellow/green) associated with the differentiation of nephrogenic mesenchyme into E-cadherin–positive (red/orange) epithelial structures (Figure 4A, red arrows). Overexpression of TAT–Cited1 inhibited branching of the UB and was associated with a reduction (Figure 4, C and D, white arrows) and enlargement (Figure 4, D and E, orange arrows) of nephronic epithelia associated with some of the UB structures. The same concentrations of recombinant TAT had no effect on epithelial patterning (Figure 4B).

Because these responses are regulated through reciprocal interactions between the condensed mesenchyme and UB epithelium, effects of exogenous Cited1 on nephrogenesis may result from direct effects on the UB epithelium and/or modification of inductive signaling by the MM. To explore this further, similar studies were performed using isolated rat MM induced to differentiate the addition of concentrated RUB1-CM. This technique is well established in our laboratory and is associated with the induction of tubular epithelial structures that can be easily identified by light microscopy (3,21,25–27). Control explants form tubular structures (Figure 4G, red arrows) that are completely absent in RUB1-CM–treated explants.
Figure 5. Cited1 is a bifunctional transcriptional regulator that activates Smad4-dependent Bmp7 signals and represses β-catenin-dependent Wnt-responses. (A) Cited1 activates Bmp7-dependent transcription. NMuMg breast epithelial cells were transiently transfected with the Bmp/TGF-β-responsive SBE-Lux reporter along with full-length HA-tagged Cited1, or deletion mutants lacking the CR2 or SID domains, as shown. Transcription of the reporter was induced by treatment with 100 ng/ml rhBmp7 for 18 h, after which cells were lysed, and luciferase activity was determined (illustrated in the horizontal bar chart). Inset shows expression of HA-tagged Cited1 constructs in the cell lysates detected by Western blot using anti-HA antibodies. (B) Cited1 represses β-catenin–dependent transcription. HepG2 cells were transiently transfected with the Wnt-responsive OT-Lux reporter along with V5-tagged Tcf4 or Lef1, and 0.25 or 0.5 µg/well of HA-tagged Cited1, as shown. (C) Cited1 represses the transcriptional activity of β-catenin. HepG2 cells were transfected with a plasmid encoding a fusion protein of Gal4 and the C-terminal transactivation domains of Xenopus β-catenin (Gal4–β-catenin R10-C, a gift from Dr Moon), along with the luciferase reporter, pG5E1B-lux, and 0.25 or 0.5 µg/well of Cited1. (D) Cited4 does not repress β-catenin–dependent transcription. HepG2 cells were transfected with the OT-Lux reporter along with V5-tagged Tcf4, and 0.5 µg/well of HA-tagged Cited1 or Cited4, as indicated. Expression levels of Cited1 and Cited4 are shown in the lower anti-HA Western blot inset. In all experiments, the constitutively active pSV–β-galactosidase reporter was cotransfected to provide a control measure of transfection efficiency. Results are expressed as the mean ± SEM of triplicate assays. Inset shows anti-HA Western blot of the transfected HepG2 cell lysates.
in the presence of TAT–Cited1 (Figure 4 H). These studies were repeated on 18 MMs: 16 of 18 treated with RUBI-CM developed tubular structures (all with >10 tubular structures/mesenchyme) compared with 0 of 18 treated with RUBI-CM and TAT–Cited1 (P < 0.01, χ² test). Transduction of a control TAT–β-galactosidase fusion protein did not block epithelial morphogenesis in this system (12 of 12 mesenchymes developed tubular structures with RUBI/CM plus TAT–β-galactosidase), indicating that repression of mesenchymal differentiation by Cited1 did not occur as a result of a nonspecific toxicity associated with TAT fusion proteins. Taken together, these findings indicate that overexpression of Cited1 blocks epithelial conversion in isolated MMs and has the capacity to inhibit branching of the UB in intact metanephroi.

Cited1 Is a Bifunctional Regulator of Smad and β-Catenin–Dependent Transcription

Our earlier studies showed that Cited1 activates TGF-β Smad4-dependent transcriptional responses (12). Because Smad4 is a common mediator of transcriptional responses downstream of other Bmp/TGF-β family ligands that are required for the regulation of diverse cellular responses within the developing kidney (6,28), we asked whether Cited1 could activate other Smad4-dependent transcriptional responses. For this, we transfected NMuMg cells with a Smad-binding element (SBE) reporter that has previously been shown to be Bmp-responsive (29) and treated the cells with recombinant Bmp7. Overexpression of Cited1 strongly activated basal and ligand-dependent reporter activity (Figure 5A). Unlike wild-type Cited1, the two Cited1 deletion mutants, Cited1 ΔSID, which lacks the Smad4 interaction domain, and Cited1 ΔCR2, which lacks the CBP/p300 interacting transactivation domain (12), did not significantly activate either basal or ligand-dependent activation of the SBE-Lux reporter. These data are consistent with the transcriptional activation of TGF-β-activated Smad4-dependent responses by Cited1 (29) and suggest that this functional property may extend to the regulation of other Smad4-dependent responses that are activated during nephrogenesis.

Wnt signaling also plays a critical role in regulating epithelial cell differentiation and UB branching during nephrogenesis (30). Because Cited1 has been shown to modify CBP and p300-dependent responses, and because these have been shown to activate canonical Wnt/β-catenin–dependent responses in mammalian cells (31–34), we sought to determine whether Cited1 might also modify Wnt signaling. We first examined the effects of Cited1 on transcriptional activation of the Wnt-responsive OT-Lux reporter (35). For these assays, we used HepG2 cells, which show basal activation of β-catenin–dependent transcription as they harbor an activating mutation of β-catenin (36). The OT-Lux reporter can be further activated by cotransfection of the β-catenin DNA-binding partners, Tcf4 or Lef1. Cited1 significantly repressed basal and Lef1/Tcf4-induced reporter gene activation (Figure 5B). Because β-catenin is required for activation of these responses, we analyzed the effects of Cited1 on β-catenin–dependent transcription directly. For this, we performed a heterologous transcription assay in which Cited1 was cotransfected along with a Gal4 fusion protein encoding the C-terminal transactivation domains of β-catenin (Gal4–β-cateninR10-C) (31), and the pG5E1b-luc reporter, which contains five Gal4-binding elements linked to a luciferase gene. Cited1 also inhibited the transactivating activity of Gal4–β-cateninR10-C (Figure 5C). These effects might result from a nonspecific sequestration of the CBP/p300 co-activators by Cited1 in these overexpression systems. We therefore tested whether Cited4, another member of the Cited family that also interacts with endogenous CBP and p300 (12,37), could suppress this response. Unlike Cited1, Cited4, if anything, activated the OT-Lux reporter (Figure 5D), suggesting that the repression of the β-catenin in these assays represents a specific functional property of Cited1.

Cited1 Inhibits β-Catenin–Dependent Transcription in Developing Xenopus Embryos

Although our cell culture data show that Cited1 has the capacity to repress β-catenin–dependent transcription, these findings may not reflect the functional properties of this protein in more complex physiologic settings. To address this, we investigated the effects of Cited1 on the formation of axial structures in Xenopus development (38). Four-cell embryos were injected in the dorsal marginal zone with Cited1 mRNA and embryos were allowed to develop in culture until stage 35. Embryos injected with Cited1 or Cited1 ΔSID mRNA showed marked impairment of axis formation, whereas Cited1 ΔCR2 did not have any effects (Figure 6, A and B, Cited1 ΔCR2 data not shown). In addition, Cited1 VP16, a chimeric construct in which the CR2 domain was replaced by the VP16 transactivator, which does not interact with CBP or p300 (39), had no effect on this response (Figure 6C), suggesting that this effect is mediated through a specific interaction between Cited1 and CBP or p300. This effect is quantified and represented graphically in the lower panels as the average score of dorsal anterior index, in which a score of five represents a normal embryo, and a score of 0 represents an embryo with complete absence of axial structures (40). The average dorsal anterior index of Cited1 and Cited1 ΔSID-injected embryos was between one and zero, whereas Cited1 VP16 yielded a score of five (Figure 6, A to C, lower panels). Histologic sections of Cited1-injected embryos revealed the disorganization of muscle tissue and the complete absence of neural tube and notochord (Figure 6, D to G). These findings suggest that Cited1 has the capacity to interfere with organizer-derived axial signals.

The signaling pathways that regulate axis formation are complex but include factors involved in the Wnt- and TGF-β/Bmp signaling pathways (38). Because Cited1 ΔSID had the same effect as full-length Cited1, inhibition of axis formation is likely to occur through a Smad4-independent mechanism. We therefore investigated the effects of Cited1 on Wnt/β-catenin–dependent responses. We first tested whether overexpression of Cited1 mRNA repressed the expression of Xnr-3, whose expression is regulated directly by β-catenin–activated transcription in Xenopus embryos (38,41). Cited1 mRNA constructs were injected into the dorsal marginal zone of 4-cell stage embryos, and Xnr3 expression was determined by whole-mount in situ
hybridization at stages nine and 10. Xnr3 expression was strongly repressed by Cited1 and Cited1 ΔSID mRNAs at stage nine, when Xnr3 expression is initiated, whereas Cited1 VP16 had no effect (Figure 6, H to K). The repression of Xnr3 expression was still observed at stage 10 (Figure 6, L to O).

To explore this further, we investigated the effects of Cited1 on the formation of Wnt/β-catenin–induced axial structures. Overexpression of β-catenin mRNA induces the formation of an ectopic organizer, which can pattern the surrounding tissue to form a secondary axis or trunk (38). Co-injection of Cited1 or Cited1 ΔSID with β-catenin mRNA inhibited this secondary axis induction, whereas co-injection of Cited1 VP16 or the Cited1 ΔCR2 deletion mutants that lack the C-terminal transactivation domain of Cited1 did not have any effect on this response (Figure 7, A to F, Cited1 ΔCR2 data not shown). These results indicate that Cited1 has the ability to repress β-catenin–dependent responses in Xenopus embryos, and that this effect depends on the C-terminal CBP/p300-interacting transactivation domain of Cited1 but does not require Smad4 interaction.

Cited1 Interacts Directly with β-Catenin in a CR2-Dependent Fashion

Inhibition of β-catenin–dependent transcription could result from a direct interaction between Cited1 and β-catenin, or may require the induction of other β-catenin repressor proteins. To explore this, we sought to determine whether Cited1 and β-catenin could interact physically. HEK 293 cells comprise a human embryonic kidney cell line that expresses molecular markers characteristic of condensed mesenchyme, including Cited1 (data not shown). Cited1 and β-catenin were immunoprecipitated from 293 cell extracts using rabbit antibodies raised against the C-terminal domain of β-catenin and Cited1 mouse monoclonal antibodies. Endogenous Cited1 immunoprecipitated with β-catenin, and β-catenin immunoprecipitated with Cited1 (Figure 8A), indicating that Cited1 forms a protein complex with β-catenin in vivo. We performed an in vitro pull-down to determine whether Cited1 binds directly to β-catenin. Glutathione-agarose beads were coated with recombinant GST or the full-length GST–β-catenin fusion protein and used as affinity matrices for in vitro–translated [35S]-methionine–labeled Cited1. Cited1 bound to β-catenin, whereas Cited1 ΔCR2 did not interact at all (Figure 8B).

Because the CR2 domain of Cited1 also interacts with the transcriptional cofactors CBP and p300 (12), we wanted to determine whether Cited1 could compete for bindings of β-catenin to p300. In vitro binding studies using GST–β-catenin and Cited1 demonstrated that both β-catenin and Cited1 bound to the C-terminal portion of p300 1135-2414 (data not shown). Using the same GST pull-down experiments, we showed that high concentrations of Cited1 competed for binding of GST–β-catenin to p300 (Figure 8C, lanes 6 to 9), whereas similar concentrations of the CR2 domain deletion mutant did not. This suggests that high concentrations of Cited1 may repress β-catenin–dependent transcription by competition for binding to the transcriptional co-activator p300.
Discussion

In this study, we have shown that Cited1 is selectively expressed in a subset of cells within the MM surrounding the UB tips, and that overexpression of Cited1 inhibits UB branching and blocks epithelial morphogenesis in renal explant cultures. Furthermore, we have shown that Cited1 acts as a bifunctional regulator of Smad4-dependent and β-catenin–dependent responses, two critical transcriptional pathways that are involved in regulating survival and differentiation of the MM. These findings suggest that Cited1 may play an important role in coordinating cellular responses during nephrogenesis.

Expression of Cited1 is restricted to the cap condensate surrounding the tip of the UB but is absent from cells within the pretubular aggregate that have undergone the second phase of patterning within the condensed mesenchyme. This pattern of expression is similar to that of the DNA-binding homeodomain factor, Six1, which is required for the MM to be competent to undergo epithelial differentiation (43), but is distinct from the expression domain of Wnt4, which is induced as these cells form the pretubular aggregates (7). Furthermore, incubation with the soluble Wnt-antagonist, Sfrp1, which we have previously shown blocks the induction of epithelial differentiation in isolated MMs (3), is associated with an expansion of Cited1 expressing cells, suggesting that there may be a reciprocal interaction between Wnt-signaling and Cited1 expression during nephrogenesis. Whether this reflects direct inhibition of Cited1 expression by Wnt signaling or an indirect effect associated with the persistence of undifferentiated mesenchyme within the explants is unknown.

Localized expression of Cited1 suggests that it may play a role in regulating the functional properties of cells within the cap condensate. Decreased expression of Cited1 as these cells form pretubular aggregates is associated with a burst of proliferation (2), suggesting that Cited1 could play a role in regulating cellular quiescence of the cap condensate and/or preventing premature nephronic differentiation. Alternatively, Cited1 may play a role in regulating inductive effects of the cap mesenchyme on branching and proliferation of cells within the UB. Our initial studies using intact rat metanephroi demonstrated that overexpression of Cited1 inhibited both processes, with a reduction and disorganization of normal nephronic differentiation associated with reduced branching of the UB system. Because nephronic induction is dependent on branching of the UB, these studies cannot determine whether the primary effects of Cited1 are on the survival and/or differentiation of cap mesenchyme, or whether they result from an inhibition of inductive signaling that is required for branching. Furthermore, although immunofluorescence studies using FITC-conjugated TAT–Cited1 indicate that this was dominantly expressed within the mesenchyme, effects on branching and nephronic induction could have resulted from the low levels of ectopic expression within the UB system. However, our studies using MMs separated from the UB indicate that Cited1 overexpression directly influences nephronic induction and/or survival of the mesenchyme, and suggest that the effects of Cited1 on UB branching in intact metanephroi could result from inhibition branching signals from the MM.

Figure 7. Cited1 blocks β-catenin–induced secondary axes in Xenopus embryos. (A to E) Secondary axes assay. β-catenin mRNA was injected into the ventral marginal zone of four-cell stage embryos along with Cited1 constructs, as indicated. Arrowheads mark induced axes. The majority of β-catenin-injected embryos developed complete secondary axes (A), whereas co-injection of Cited1 with β-catenin mRNA suppressed the formation of complete axes, but some partial axes were induced (B). Higher doses of Cited1 completely inhibited ectopic axes induced by β-catenin (C). Cited1ΔSID can efficiently inhibit β-catenin, whereas Cited1 VP16 is ineffective (D, E). (F) Secondary axes induction assay graphs. N, normal; P, partial; C, complete axes induced. Complete axes were scored as axes containing cement gland and two eyes as shown in (A), whereas partial axes were embryos that exhibited ectopic trunk structures as shown in (B).
To determine how Cited1 might be regulating functional properties of the cap mesenchyme, we looked at the effects of Cited1 on transcriptional responses that are known to control cellular survival and differentiation in the MM. Our findings suggest a potential mechanism, because Bmp7-dependent activation of Smad4 plays a critical role in recruitment and survival of the cap mesenchyme (6). On this basis, misexpression of Cited1 might be expected to enhance Bmp7-dependent cellular survival and promote an expansion of the cap mesenchyme. However, our overexpression studies indicate that Cited1 has the
capacity to block nephronic induction, and there is no evidence that Smad4 signaling inhibits cellular differentiation of the condensed mesenchyme.

We have shown that epithelial differentiation of cultured MM is associated with activation of the canonical Wnt/β-catenin pathway (25) and that this response can be blocked by incubating the mesenchymes with the secreted Wnt antagonist, Sfrp1 (3). Furthermore, Wnt signaling has been shown to play a critical role in regulating growth and branching of the UB (30), suggesting that modification of these signaling pathways in renal explant cultures could account both for the effects of Cited1 on branching of the UB and differentiation of the cap mesenchyme. Unlike the effects on Smad4 signaling, our studies demonstrate that Cited1 has the capacity to inhibit β-catenin–dependent transcriptional responses in a variety of different biologic systems and suggest that this effect is a result of direct competition by Cited1 for binding between p300 and β-catenin. This indicates that Cited1 is a bifunctional transcriptional regulator, activating Smad4-dependent transcriptional responses through the recruitment of CBP and p300 (12) while inhibiting β-catenin–dependent responses by direct competition for p300. Other members of the Cited family have similar bifunctional CBP/p300-dependent effects on different transcriptional responses. For example, Cited2 and 4 interact with and activate Tfap-2–dependent responses (44,45) while inhibiting Hif-1α–dependent transcription by direct competition for binding to CBP and p300 (18,46). These findings raise the possibility that Cited1 may play a central role in coordinating diverse signaling pathways that are activated in and around the cap mesenchyme. Because Cited1 is downregulated in the pre-tubular aggregates that express high levels of Wnt4, and because Wnt4 has the capacity to autoregulate its expression through a positive feedback loop (7), expression of Cited1 in the cap mesenchyme may serve to limit the spread Wnt4 expression at the junction of the pretubular aggregates and cap mesenchyme, thereby inhibiting premature Wnt-dependent nephric induction in the cap condensates. At the same time, activation of Smad4-dependent responses by Cited1 may enhance Bmp7–dependent recruitment and survival of cells within the cap condensate.

In summary, we have shown that Cited1 is expressed in cap condensates and has the capacity inhibit branching of the UB and epithelial cell differentiation of the MM. Furthermore, we have shown for the first time that Cited1 has the capacity to act as a bifunctional transcriptional regulator, repressing Wnt/β-catenin signaling while at the same time stimulating Bmp and TGF-β–activated Smad4-dependent transcriptional responses. On this basis, we propose that Cited1 may regulate cellular function in the MM by providing a transcriptional switch that is necessary to coordinate the convergent signals that cooperate to regulate cellular survival and differentiation in the developing kidney.

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