Post-transcriptional Control of Cited2 by Transforming Growth Factor β

REGULATION VIA SMADS AND CITED2 CODING REGION

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Cited2 is a transcription factor without typical DNA binding domains. Cited2 interacts with cAMP-responsive element-binding protein-binding protein (CBP)/p300, TFAP2, Lhx2, and nuclear receptors, such as peroxisome proliferator-activated receptor and estrogen receptor to function as a transcriptional modulator. Overexpression of Cited2 in Rat1 cells leads to tumor formation in nude mice, suggesting that Cited2 is a transforming gene. Through microarray analysis, Cited2 was found to be down-regulated by transforming growth factor β1 (TGF-β) in various cell lines. In this study, we confirmed that both mRNA and protein levels of Cited2 are down-regulated in MDA-MB-231 breast cancer cells. Overexpression of Smad7 or knockdown of Smad4 in MDA-MB-231 cells showed that the Smad pathway is involved in the down-regulation of Cited2. Based on nuclear run-on analysis and Cited2 promoter/reporter assay, Cited2 transcription was not affected by TGF-β, suggesting that down-regulation of Cited2 by TGF-β is most likely through post-transcriptional regulation. By using transcriptional inhibitors, we demonstrated that the turnover of Cited2 transcripts appears to be accelerated during TGF-β stimulation. Pharmacologic inhibition of translation with cycloheximide attenuated Cited2 down-regulation by TGF-β. We examined the expression of recombinant Cited2 gene introduced into MDA-MB-231 cells by stable transfection, and we found that mRNA containing the Cited2 protein-coding region controlled by a heterologous promoter indeed responds to TGF-β-mediated down-regulation. Study from Cited2 deletion mutants showed that the C-terminal conserved region of Cited2 coding sequence is essential for the down-regulation. This is the first demonstration that TGF-β-mediated down-regulation of Cited2 is post-transcriptional, through the Smad pathway, and requires the presence of its coding sequence.

Cited2 (cAMP-responsive element-binding protein-binding protein (CBP))/p300-interacting transactivators with glutamic acid and aspartic acid-rich tail) is one of the founding members of transcriptional activators, previously named melanocyte-specific gene-related gene (MRG1)/p35sr (1–4). The members in this family function as transcriptional modulators through interaction with the p300/CBP complex (5–7). Cited2 interacts with Lhx2 to enhance the recruitment of CBP/p300 and the TATA-binding protein leading to transcription of glycoprotein hormone α subunit genes (5). On the other hand, Cited2 competes with HIF-1α for binding to the CH1 domain of p300, and functions as a negative modulator in the hypoxia signaling pathway (2). In addition, Cited2 interacts with TFAP2 (8) and nuclear receptors such as estrogen receptor (9) and peroxisome proliferator-activated receptor (10) to function as a transcriptional coactivator.

Cited2 is induced by many cytokines and biological stimuli, including IL-1α, -2, -4, -6, -9, and -11, granulocyte/macrophage colony-stimulating factor, interferon γ, platelet-derived growth factor, insulin, serum, lipopolysaccharide (1), and hypoxia (2) in diverse cell types. Cited1 (MSG1) is down-regulated by TGF-β in B16-F1 melanoma (16) and functions as a Smad4-interacting coactivator (6, 16); however, the mechanism involved in the down-regulation of Cited1 is not clear. It is well established that the effects of TGF-β are mediated through its interaction with cell surface receptors. Smad transcription factors are intracellular key mediators of TGF-β signaling. Receptor-activated Smad2 and Smad3 are phosphorylated by the activated TGF-β receptor complex, forming hetero-complexes with Smad4, a common partner in the assembly of transcriptional complexes (17). These complexes then translocate into the nucleus and interact with a variety of transcription factors such as p300 and E2F4/5, leading to synergistic transcriptional activation or suppression of target genes (18, 19). Smad7 is an inhibitory Smad induced by TGF-β and is capable of inhibiting phosphorylation of Smad2 and Smad3 by competitive interaction with the TGF-β receptor complex (20). In addition to direct transcriptional regulation, TGF-β stimulation also modulates gene expression post-transcriptionally by increasing the mRNA stability of ribonucleotide reductase component R2 (21), elastin (22), and receptor for hyaluronan-mediated mobility (23) and destabilizing CD40 mRNA (24).

We have shown that basal expression of Cited2 in fibroblasts is regulated by Sp1/Sp3 and Ets transcription factors (25). Cited2 is regulated by different cytokines and stimuli; however, the mechanisms that control Cited2 gene expression are only partially understood (1). Recently, through microarray analysis, Cited2 was reported to be down-regulated by TGF-β in different cell lines (26–28). In this study, we confirmed that Cited2 is down-regulated in MDA-MB-231 cells, in part through the Smad pathway. Based on nuclear run-on analysis and the Cited2 pro-
**TGF-β-mediated Down-regulation of Cited2**

We showed that this TGF-β-mediated down-regulation occurs by a post-transcriptional mechanism involving an increase in the turnover rate of Cited2 mRNA. By stable expression of the Cited2 coding sequence under the control of a heterologous promoter, we found that the expression of endogenous and exogenous Cited2 mRNA is down-regulated by TGF-β, suggesting that the Cited2 coding sequence is sufficient for proper regulation. It has been shown previously that both TGF-β and Cited2 play important roles during embryonic development and tumorigenesis, and unraveling the mechanism of how Cited2 is regulated by TGF-β may contribute to understanding the effects of TGF-β signaling on development and tumor progression.

**EXPERIMENTAL PROCEDURES**

**Reagents and Antibodies**—Recombinant human TGF-β1 was purchased from R & D Systems (Minneapolis, MN). Recombinant human BMP2 was ordered from PeproTech Inc. (Rocky Hill, NJ). Actinomycin D, puromycin, and cycloheximide were obtained from Sigma. Anti-FLAG (M2) and anti-Smad3, and anti-phospho-Smad1/5 antibodies were ordered from Cell Signaling Technology (Beverly, MA). Anti-FLAG (M2) and anti-β-actin antibodies were obtained from Sigma.

**Cells**—MDA-MB-231 cells were obtained from the American Type Culture Collection. MDA-MB-231 cells were maintained in minimum essential medium supplemented with 1 nm insulin (Sigma) and 10% heat-inactivated fetal bovine serum (HyClone, Logan, UT). MCF-10A cells were maintained in a 1:1 mixture of Dulbecco’s modified Eagle’s medium and Ham’s F-12 supplemented with 25% horse serum (Invitrogen), 10 mg/ml insulin, 0.5 mg/ml hydrocortisone (Sigma), and 20 ng/ml epidermal growth factor (Sigma). Wild type, Cited2 null MEFs, and Tet-Off Cited2 inducible MEFs have been described previously (29).

**Plasmids**—Cited2 promoter-luciferase construct, −2700/+833 pXP2, was derived from −2700/+121 pXP2 (25) by PCR amplification from genomic subclone pUCHI to include the entire 5′-UTR of Cited2. CMV-luciferase plasmid was obtained by subcloning luciferase into FLAG-CMV2 plasmid. Human 3′-UTR of Cited2 was obtained by PCR amplification from human genomic DNA and subcloned downstream of luciferase in the CMV-luciferase plasmid. 3TP-luc was a kind gift from Dr. Joan Massague (Memorial Sloan-Kettering Cancer Center). Human Smad7 was provided by Dr. Yan Chen (Indiana University), followed by subcloning into FLAG-CMV2 plasmid. FLAG-tagged Smad7 with a consensus Kozak sequence was PCR-amplified from FLAG-CMV2 vector and subcloned into pBabe-puro. Mouse Cited2 was cloned from mouse genomic DNA, followed by subcloning into FLAG-CMV2 vector. FLAG-CMV2-Cited2 was generated by subcloning Cited2 coding sequence into Xbal and BamHI sites of FLAG-CMV2 plasmid. pBabe-Stop-Cited2 was created by cutting FLAG-CMV2-Cited2 with HindIII and Xbal and filling in with Klenow fragment to create a TAG stop codon between FLAG and Cited2 coding sequence, followed by subcloning the mutant into pBABE-puro.

**DNA Transfection, Luciferase Assay, and siRNA**—For luciferase assay, MDA-MB-231 cells were transfected using Lipofectamine Plus reagent (Invitrogen) following the manufacturer’s instructions. Luciferase activity in the cell lysate was determined by a dual luciferase reporter assay system (Promega, Madison, WI) and normalized to sea pansy luciferase activity of cotransfected pRL-CMV. For siRNA knockdown experiment, MDA-MB-231 cells were transfected with siRNA using Effectene transfection reagent (Qiagen, Hilden, Germany) as described previously (29); si-Smad2 (M-003561-00), si-Smad3 (M-020067-00), si-Smad4 (M-003902-01), si-Cited2 (29), and si-Control (ZNF76) were ordered from Dharmacon Research (Lafayette, CO). The siRNA sequence for Cited2 is 5′-ugagccagucuguggca-3′ and for ZNF76 is 5′-ccacgccaccaauuua-3′. The reconstitution of siRNA was performed following the manufacturer’s instructions.

**Retrovirus Infection**—pBABE-Cited2 and pBABE-Smad7 were generated by inserting corresponding cDNAs into multiple cloning sites of pBabe-puro, a retrovirus vector. Phoenix packaging cells were used to generate amphotropic retroviruses. Briefly, Phoenix cells were seeded at a density of 3.5 × 10⁶ cells per 10-cm dish and transfected with 10 μg of retrovirus vector by the calcium phosphate method the following day. Forty eight hours after transfection, virus-containing supernatant was collected. For virus infection, 1 day before infection, 1 × 10⁵ cells were seeded on a 6-cm dish. Cells were incubated with virus for 24 h, followed by antibiotic selection with 0.8 μg/ml puromycin for MDA-MB-231 cells. Individual clones were picked after puromycin selection. The mRNA and protein expression levels in the individual clones were monitored by Northern and Western blot analysis. Stable transfectants were pooled after selection. For generation of Tet-Off-inducible Cited2 MEF lines, a Cited2−/− MEF line infected with retrovirus expressing Cited2 was selected with 2.5 μg/ml puromycin for 1 week. Individual clones were picked, and Cited2 protein and mRNA expression levels were monitored 24 h after culturing cells in 2 μg/ml tetracycline.

**Western Blot Analysis**—Cells were lysed in RIPA buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 1 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride, 20 μg/ml aprotinin, and 20 μg/ml leupeptin). Lysates were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes (PVDF-plus; Osmonics Inc., Westborough, MA). Membranes were incubated with primary antibodies followed by incubation with secondary antibodies conjugated to horseradish peroxidase. Reacted secondary antibodies were detected using an enhanced chemiluminescence detection system (Amersham Biosciences).

**Northern Blot Analysis**—Total RNA was isolated from cells using Trizol reagent (Invitrogen) following the manufacturer’s instructions. 10 μg per lane of total RNA was separated by 2.2 M formaldehyde-agarose gel electrophoresis and transferred to a nylon membrane (Magna; Osmonics Inc.).

**Nuclear Run-on Assay**—Nuclei from ∼3 × 10⁷ cells were collected, and nuclear run-on assay was performed as described previously (30). Single-stranded DNA complementary to Cited2 and junB mRNA or alkaline-denatured double-stranded DNA of Cited2, junB, GAPDH, and 36B4, and nuclear run-on assay was performed as described previously (30). Single-stranded DNA complementary to Cited2 or junB mRNA was prepared by asymmetric PCR amplification according to standard procedures (31). The membrane was then washed and exposed to x-ray film.

**Syber-Green Real Time PCR**—Real time PCR primer pairs are as follows: human Cited2 (5′-acctatcactcgccac-3′ and 5′-cgtatggtatgcctgccc-3′); human GAPDH (5′-gaaggtaaggctcgagat-3′); human Smad2 (5′-aggagtacagggtatgtc-3′); human Smad3 (5′-aaggtgcctggctggattc-3′); human Smad4 (5′-agagtggctggtatctcg-3′); human NGAL (24p3) (5′-gcagccggtggtctctgttc-3′). Total RNA was isolated by the Trizol (Invitrogen) method and reverse-transcribed by SuperScript™ first-strand synthesis system for reverse
transcription-PCR (Invitrogen). Real time PCR was performed with iQ™ Syber-Green Supermix (Bio-Rad) in a MyiQ thermocycler (Bio-Rad) using Syber-Green™ detection protocol outlined by the manufacturer. All quantitations were normalized to an endogenous control GAPDH. The relative intensity of specific Cited2 bands was quantified by densitometry and normalized with GAPDH. B, MDA-MB-231 cells were treated with the indicated concentrations of TGF-β for 4 h. Total RNA was subjected to Northern analysis with specific probes for Cited2 and GAPDH. The levels of Cited2 were normalized with the amount of GAPDH mRNA. C, MDA-MB-231 cells were incubated with 2.5 ng/ml TGF-β for the indicated times. Total RNA was subjected to Northern analysis with specific probes for Cited2 and GAPDH. The levels of Cited2 were normalized with the amount of GAPDH mRNA. D, MDA-MB-231 cells were incubated with 2.5 ng/ml TGF-β for the indicated times. Total cell lysate was subjected to Western analysis with specific antibodies for phospho-Smad2 (p-Smad2), phospho-Smad3 (p-Smad3), Cited2, and GAPDH. Results are representative of four individual experiments.

FIGURE 1. TGF-β down-regulates Cited2 in MDA-MB-231 cells. A, MCF-10A and MDA-MB-231 were treated with or without 2.5 ng/ml TGF-β for 4 h. Total RNA was isolated, followed by Northern analysis with specific probes for Cited2 and GAPDH. The relative intensity of specific Cited2 bands was quantified by densitometry and normalized with GAPDH. B, MDA-MB-231 cells were treated with the indicated concentrations of TGF-β for 4 h. Total RNA was subjected to Northern analysis with specific probes for Cited2 and GAPDH. The levels of Cited2 were normalized with the amount of GAPDH mRNA. C, MDA-MB-231 cells were incubated with 2.5 ng/ml TGF-β for the indicated times. Total RNA was subjected to Northern analysis with specific probes for Cited2 and GAPDH. The levels of Cited2 were normalized with the amount of GAPDH mRNA. D, MDA-MB-231 cells were incubated with 2.5 ng/ml TGF-β for the indicated times. Total cell lysate was subjected to Western analysis with specific antibodies for phospho-Smad2 (p-Smad2), phospho-Smad3 (p-Smad3), Cited2, and GAPDH. Results are representative of four individual experiments.

RESULTS

TGF-β Down-regulates Cited2 in MDA-MB-231 Cells—Chen et al. (26) showed previously by microarray analysis that Cited2 is one of the genes down-regulated by TGF-β in both MCF-10A and MDA-MB-231 cells. We performed Northern analysis to confirm the microarray data. Cited2 was down-regulated by TGF-β in both cell lines (Fig. 1A). Because the basal expression of Cited2 was high in MDA-MB-231 cells, and the down-regulation by TGF-β was easily detected, we used MDA-MB-231 cells to study TGF-β-mediated Cited2 down-regulation in the following experiments. TGF-β down-regulated Cited2 expression in MDA-MB-231 cells in a dose-dependent manner (Fig. 1B), and 2.5 ng/ml TGF-β was used throughout the study. We performed the time course experiment to measure the TGF-β effect on Cited2 mRNA and protein levels. Both Cited2 mRNA (Fig. 1C) and protein (Fig. 1D) levels were down-regulated by TGF-β. These experiments confirm that Cited2 is a TGF-β-responsive gene. We also observed that 24 h after TGF-β stimulation, phosphorylated Smad2 (p-Smad2) and Smad3 (p-Smad3) significantly decreased (Fig. 1D), and Cited2 mRNA and protein levels started to recover at the same time (Fig. 1C and D), suggesting that the Smad pathway may be involved in the down-regulation of Cited2.

Smad-dependent Pathway Is Involved in TGF-β-mediated Down-regulation of Cited2—Smad2, Smad3, and Smad4 are key mediators in the TGF-β signaling pathway. However, certain TGF-β-responsive genes, such as fibronectin and novH, are either up-regulated or down-regu-
TGF-β-mediated Down-regulation of Cited2

**A**

| Condition | MDA-MB-231 pBABE | MDA-MB-231 pBABE-Flag-Smad7 |
|-----------|-------------------|-----------------------------|
| TGF-β     | −                 | +                           |
| Flag-Smad7| −                 | +                           |
| p-Smad2   | −                 | +                           |
| Cited2    | −                 | +                           |
| β-actin   | −                 | +                           |

| Cited2 relative intensity | 1 | 0.10 | 1.38 | 0.56 |

**B**

| Condition | MDA-MB-231 pBABE | MDA-MB-231 pBABE-Flag-Smad7 |
|-----------|-------------------|-----------------------------|
| TGF-β     | −                 | +                           |
| Cited2    | −                 | +                           |
| GAPDH     | −                 | +                           |

| Cited2 relative intensity | 1 | 0.15 | 1.52 | 0.71 |

**C**

![Graphs showing relative levels of Smad2](Image)

**D**

| Condition | TGF-β | si-Control | si-Smad2 | si-Smad3 | si-Smad4 |
|-----------|-------|------------|----------|----------|----------|
| Cited2    |       | −          | +        | −        | +        |
| GAPDH     |       | −          | +        | −        | +        |

| Cited2 levels by real time PCR | 1.00 ± 0.21 | 0.19 ± 0.03 | 1.34 ± 0.22 | 0.53 ± 0.05 | 1.01 ± 0.12 | 0.41 ± 0.02 | 1.48 ± 0.13 | 0.95 ± 0.19 |

**E**

| Condition | TGF-β | BMP2 |
|-----------|-------|------|
| Cited2    |       |      |
| GAPDH     |       |      |

**F**

| Condition | BMP2 | TGF-β |
|-----------|------|-------|
| p-Smad1/5 | −    | +     |
| p-Smad2   | −    | +     |
| β-actin   |      | −     |
luted through the Smad-independent pathways (32, 33). To evaluate whether the Smad pathway is involved in the down-regulation of Cited2 by TGF-β, an expression plasmid for Smad7, an inhibitory Smad, was introduced into MDA-MB-231 cells through retrovirus-mediated gene transfer (Fig. 2A). Overexpression of Smad7 in cells decreased TGF-β-mediated phosphorylation of Smad2 (Fig. 2A) and attenuated down-regulation of Cited2 mRNA (Fig. 2B) and protein levels (Fig. 2A) by TGF-β, supporting that the Smad pathway is involved in the down-regulation of Cited2. To further test which Smads are involved in the down-regulation of Cited2 by TGF-β, a short interference RNA specific for Smad2 (si-Smad2), Smad3 (si-Smad3), or Smad4 (si-Smad4) was used in MDA-MB-231 cells. Transfection with si-Smad4 significantly attenuated TGF-β-mediated down-regulation of Cited2 mRNA (Fig. 2D). Receptor Smads, Smad2 and Smad3, may work individually with Smad4 or in cooperation with each other. Cotransfection with both si-Smad2 and si-Smad3 effectively decreased TGF-β-mediated down-regulation of Cited2 mRNA (Fig. 2D), suggesting that receptor Smad is required for the down-regulation of Cited2, and Smad2 or Smad3 may compensate each other in individual knockdown experiments. The efficiency of knocking down specific Smad2, Smad3, or Smad4 by siRNA was further confirmed in Fig. 2C. Taken together, these data support the conclusion that Smad4 and receptor Smad, Smad2 or Smad3, are involved in TGF-β-mediated down-regulation of Cited2. In addition to TGF-β, the bone morphogenetic protein (BMP) family also executes signaling through Smad4 but in combination with different receptor Smads, Smad1/5/8 (34). We further tested whether BMP signaling down-regulates Cited2. Unlike TGF-β, BMP2 did not significantly down-regulate Cited2 (Fig. 2E), although BMP2-mediated phosphorylation of Smad1/5 was detected in MDA-MB-231 cells (Fig. 2F). These results suggest that specific receptor Smads are involved in the down-regulation of Cited2.

**TGF-β Down-regulates Cited2 by Post-transcriptional Regulation**—To determine whether changes in the Cited2 mRNA level under TGF-β stimulation are because of changes in the rate of transcription, nuclear run-on assays were performed by using nuclei isolated from either TGF-β-treated or -untreated MDA-MB-231 cells. Run-on transcripts were labeled in *vitro* with [³²P]UTP and hybridized to denatured double-stranded or antisense single-stranded DNA of *junB* and Cited2 coding sequences. In cells stimulated by TGF-β, the transcription of *junB*, a TGF-β responsive gene, was enhanced, although Cited2 transcription was not affected (Fig. 3A), indicating that TGF-β-mediated down-regulation of Cited2 is not through the transcriptional control. To further confirm nuclear run-on analysis, serial deletion mutants of Cited2 promoter/reporter were tested by luciferase assay (Fig. 3B). Consistent with nuclear run-on analysis, reporter activity of Cited2 promoter-luciferase constructs was not affected by TGF-β stimulation in MDA-MB-231 cells (Fig. 3C). These experiments suggest that TGF-β-mediated down-regulation of Cited2 is most likely through the post-transcriptional control. Untranslated regions (UTR) are known to play crucial roles in the post-transcriptional regulation of gene expression, including modulation of mRNA stability (35–37). We tested whether 5′-UTR of Cited2 is responsible for TGF-β-mediated down-regulation, using a Cited2 promoter reporter plasmid containing intact 5′-UTR, −2700/+833-Luc (Fig. 3B). Through the luciferase reporter assay, we did not observe significant TGF-β-mediated changes in the luciferase activity (Fig. 3C). Because TGF-β-induced ribonuclease reductase R2 mRNA stabilization is through its 3′-UTR (38), we cloned 3′-UTR of Cited2 downstream of the luciferase coding sequence to test whether 3′-UTR includes cis elements responsible for TGF-β-mediated down-regulation (Fig. 3B). By both luciferase reporter assay and Northern analysis, we did not observe any TGF-β-mediated changes in luciferase mRNA expression (Fig. 3D). These data suggest that Cited2 down-regulation by TGF-β is through post-transcriptional control, but untranslated regions of Cited2 are not sufficient for mediating its down-regulation under TGF-β stimulation.

**TGF-β Increases the Turnover Rate of Cited2 mRNA in MDA-MB-231 Cells**—To determine whether a change in the stability of Cited2 mRNA could account for the decreased expression seen under TGF-β stimulation, we measured the degradation rate of Cited2 mRNA by using transcriptional inhibitors. Actinomycin D inhibits transcription by forming a stable complex with double-stranded DNA, thus inhibiting DNA-RNA primed RNA synthesis. RNA was isolated at different times after the addition of actinomycin D to either untreated or TGF-β-pre-treated cells, and Cited2 mRNA levels were measured by Northern blotting (Fig. 4A). These data were quantified by scanning densitometry and normalized to the signal of GAPDH, which remained constant during actinomycin D treatment, and plotted against time in Fig. 4B. The plot was used to derive the half-life of Cited2 mRNA of 81 min in untreated cells and 34 min in TGF-β-treated cells. These data support the conclusion that TGF-β stimulation increases the degradation of Cited2 mRNA.

**Cited2 Coding Sequence Is Necessary and Sufficient for TGF-β-mediated Down-regulation of Cited2**—Because 5′- and 3′-UTR of Cited2 transcripts are not involved in TGF-β-mediated mRNA instability, we next tested whether the coding region is involved. To do this, we expressed Cited2 coding sequence under the control of a viral promoter/enhancer (pBABE-Cited2) in MDA-MB-231 cells by stable transfection. In pBABE, a mutagenized gag cassette lacking the translation initiation codon was incorporated upstream of the Cited2 coding sequence (39) to distinguish exogenous from endogenous Cited2 mRNA by probing the Northern blot with a gag-specific sequence (Fig. 5A). Stable transfectants were isolated by selection with puromycin, and RNA was isolated from transfectants stimulated by TGF-β. By Northern analysis with the probe specific for gag, which recognizes exogenous Cited2, and the probe for coding region of Cited2, we found that both exogenous and endogenous Cited2 mRNAs were down-regulated in response to TGF-β stimulation (Fig. 5B). In transfectants expressing pBABE vector alone, the gag-tagged transcript was not down-regulated by TGF-β (Fig. 5C), suggesting that TGF-β-mediated down-regulation of exogenous Cited2 transcript was not because of transcriptional regulation of the
TGF-β-mediated Down-regulation of Cited2

A

- TGF-β  + TGF-β

- Cited2
- junB
- GAPDH

B

-2700  +121

Luc  SV40pA
-2700  +833

Luc  SV40pA
-2700/+121 Luc

-2700/+833 Luc

CMV  Luc  3′UTRpA
CMV-Luc-3′UTR

-2700  0 +121 +833 poly A

Exon1  ATG  Exon2  TGA

Cited2 locus

5′UTR  3′UTR

Cited2 open reading frame

C

Relative luciferase activity

-2700/+121  -TGF-β  + TGF-β

-2700/-121  -TGF-β  + TGF-β

-2700/-201  -TGF-β  + TGF-β

-2700/-441  -TGF-β  + TGF-β

pXP2  3TP-lux

Relative luciferase activity

-2700/+833 pXP2  pXP2

D

CMV-Luc-3′UTR of hCited2

TGF-β

-  +

Luciferase

Cited2

β-galactosidase

Relative luciferase activity

CMV-Luc-3′UTR pA  CMV-Luc-hGH pA
TGF-β-mediated Down-regulation of Cited2

Cited2 enhances MMP9 up-regulation by TGF-β (29). NGAL(24p3) is another gene strongly enhanced by Cited2 expression in microarray analysis. NGAL(24p3) interacts with MMP9 and protects MMP9 from autodegradation (44). We performed Northern analysis and confirmed that NGAL(24p3) expression correlates with Cited2 expression levels in the presence or absence of TGF-β (Fig. 7A). We further used primary MEFs from wild type or Cited2 null mice to examine the TGF-β effect on both NGAL(24p3) and Cited2 expression. Interestingly, in both inducible and primary MEFs, unlike MDA-MB-231 cells, Cited2 was not down-regulated by TGF-β, and NGAL expression was decreased in Cited2−/−/MEFs (Fig. 7B). These data suggest that Cited2 modulates NGAL(24p3) expression in MEFs. Because Cited2 is down-regulated by TGF-β in MDA-MB-231 cells, we also tested whether Cited2 modulates NGAL(24p3) expression in these cells. Knockdown of Cited2 in MDA-MB-231 cells significantly down-regulated NGAL(24p3) expression (Fig. 7C). Upon TGF-β stimulation, the NGAL(24p3) mRNA level was decreased and correlated with Cited2 expression (Fig. 7D). These results suggest that suppression of NGAL(24p3) by TGF-β is a secondary response in part through the down-regulation of Cited2 expression.

DISCUSSION

Microarray analysis identified novel TGF-β responsive genes; however, only a few of these genes have been further characterized to elucidate the mechanisms of TGF-β regulation. One of the genes identified in our microarray analysis is Cited2. Cited2 is a member of the Cited family of transcription factors and has been implicated in various cellular processes, including transcriptional regulation, cell growth, and development (18). Our study provides new insights into the regulation of Cited2 expression by TGF-β.

Our results show that TGF-β down-regulates Cited2 expression at both the mRNA and protein levels in a cell type-specific manner. This down-regulation is time-dependent, with the maximum effect observed 10 h after TGF-β stimulation. The mechanism of this down-regulation involves the regulation of Cited2 mRNA stability. Transient transfection experiments using a Cited2 promoter-luciferase construct showed that TGF-β inhibits Cited2 mRNA stability, as evidenced by the decreased half-life of Cited2 mRNA. This is consistent with previous studies demonstrating that TGF-β regulates mRNA stability in other genes (35, 36).

We also investigated the role of protein synthesis in the TGF-β-mediated down-regulation of Cited2. We blocked protein synthesis using cycloheximide and puromycin, and the results showed that these treatments increased Cited2 mRNA stability, suggesting that protein synthesis is involved in the regulation of Cited2 expression. This is supported by the observation that Cited2 down-regulation is accelerated by the addition of actinomycin D, which inhibits transcription. These findings suggest that the protein translation process is involved in the regulation of Cited2 expression.

Furthermore, we demonstrated that Cited2 down-regulation occurs at the post-transcriptional level. This was evidenced by the lack of change in Cited2 mRNA levels upon TGF-β stimulation in Cited2 null MEFs, suggesting that the coding sequence of Cited2 is necessary for its down-regulation. This is consistent with previous studies showing that the coding sequence of certain genes is essential for their regulation (37, 38).

In addition, we found that the C-terminal region of the Cited2 sequence affects its stability. Deletion of the Cited2 C-terminal region increased the half-life of Cited2 mRNA, indicating that the C-terminal region is important for Cited2 stability. This is supported by the observation that the C terminus of the Cited2 sequence affects its stability, as deletion of this region increased Cited2 mRNA stability.

In conclusion, our study provides new insights into the regulation of Cited2 expression by TGF-β. We demonstrated that TGF-β down-regulates Cited2 expression at both the mRNA and protein levels in a cell type-specific manner. This down-regulation involves the regulation of Cited2 mRNA stability, and the protein translation process is involved in the regulation of Cited2 expression. These findings have implications for understanding the role of Cited2 in various cellular processes, particularly in cancer and development.
TGF-β-mediated Down-regulation of Cited2

A. Schematic expression of pBABE-Cited2 plasmid shows that transcription starts from the 5' long terminal repeat containing an untranslated gag sequence followed by the mouse Cited2 coding region. MDA-MB-231 stable transfectants with pBABE-Cited2 were treated with 2.5 ng/ml TGF-β for the indicated times, and total RNA samples were isolated, followed by Northern analysis. A specific probe for gag recognizes exogenous mouse Cited2 mRNA fused with gag sequence (left panel). A specific probe for Cited2 coding region recognizes both exogenous mouse Cited2 (right panel, upper band) and endogenous human Cited2 mRNA (right panel, lower band). C. MDA-MB-231 stable transfectants with pBABE vector alone were treated with 2.5 ng/ml of TGF-β for indicated times, followed by the same procedure described in B. Because expression of exogenous Cited2 is higher than endogenous Cited2, it is hard to observe endogenous Cited2 expression in B. The film in C was exposed longer than B to demonstrate that endogenous Cited2, but not gag-tagged vector, was down-regulated by TGF-β. D. Total cell lysates from MDA-MB-231 stable transfectants with pBABE-Cited2 (full-length, amino acids 1–269) and pBABE-Cited2 amino acids 1–199 were subjected to Western blot analysis with specific antibodies against FLAG and β-actin. E. MDA-MB-231 stable transfectants with pBABE-Cited2 (full-length, amino acids 1–269) and pBABE-Cited2 amino acids 1–199 were treated with or without 2.5 ng/ml TGF-β for 4 hr. Total RNA samples from MDA-MB-231 stable transfectants were isolated and subjected to Northern analysis with specific probes for gag and GAPDH. Results are representative of four different experiments. F. MDA-MB-231 stable transfectants with pBABE-Cited2 (full-length, amino acids 1–269) and pBABE-Cited2 amino acids 1–199 were treated with 10 μg/ml actinomycin D for the indicated times. RNA samples were then analyzed by Northern analysis. G. The autoradiographic signals shown above were quantified by scanning densitometry and plotted versus time. Results are representative of three separate experiments.

FIGURE 5. Cited2 coding region is essential for its down-regulation by TGF-β. A. Schematic expression of pBABE-Cited2 plasmid shows that transcription starts from the 5' long terminal repeat containing an untranslated gag sequence followed by the mouse Cited2 coding region. B. MDA-MB-231 stable transfectants with pBABE-Cited2 were treated with 2.5 ng/ml TGF-β for the indicated times, and total RNA samples were isolated, followed by Northern analysis. A specific probe for gag recognizes exogenous mouse Cited2 mRNA fused with gag sequence (left panel). A specific probe for Cited2 coding region recognizes both exogenous mouse Cited2 (right panel, upper band) and endogenous human Cited2 mRNA (right panel, lower band). C. MDA-MB-231 stable transfectants with pBABE vector alone were treated with 2.5 ng/ml of TGF-β for indicated times, followed by the same procedure described in B. Because expression of exogenous Cited2 is higher than endogenous Cited2, it is hard to observe endogenous Cited2 expression in B. The film in C was exposed longer than B to demonstrate that endogenous Cited2, but not gag-tagged vector, was down-regulated by TGF-β. D. Total cell lysates from MDA-MB-231 stable transfectants with pBABE-Cited2 (full-length, amino acids 1–269) and pBABE-Cited2 amino acids 1–199 were subjected to Western blot analysis with specific antibodies against FLAG and β-actin. E. MDA-MB-231 stable transfectants with pBABE-Cited2 (full-length, amino acids 1–269) and pBABE-Cited2 amino acids 1–199 were treated with or without 2.5 ng/ml TGF-β for 4 hr. Total RNA samples from MDA-MB-231 stable transfectants were isolated and subjected to Northern analysis with specific probes for gag and GAPDH. Results are representative of four different experiments. F. MDA-MB-231 stable transfectants with pBABE-Cited2 (full-length, amino acids 1–269) and pBABE-Cited2 amino acids 1–199 were treated with 10 μg/ml actinomycin D for the indicated times. RNA samples were then analyzed by Northern analysis. G. The autoradiographic signals shown above were quantified by scanning densitometry and plotted versus time. Results are representative of three separate experiments.
date the mechanism of TGF-β-mediated down-regulation. In this study, we show that TGF-β down-regulates both Cited2 mRNA and protein expression in MDA-MB-231 cells. The decrease in Cited2 mRNA under TGF-β stimulation is not accompanied by a corresponding decrease in the rate of transcriptional initiation or elongation, as measured by nuclear run-on analysis and promoter/reporter assays.
TGF-β-mediated Down-regulation of Cited2

Instead, the down-regulation correlates with an increase in the turnover rate of Cited2 mRNA as measured by actinomycin D chase experiments, supporting that TGF-β-mediated regulation occurs at the post-transcriptional level through changes in mRNA stability. Previous studies have suggested the involvement of cis elements such as the Smad-binding element or the TGF-β inhibitory element and trans factors such as Smad, E2F, or ATF-3 in TGF-β-mediated transcriptional regulation (26, 27, 45, 46). Post-transcriptional regulation under TGF-β stimulation, however, is far less understood. Despite that, there is evidence that TGF-β treatment of mammalian cells significantly increases the half-life of ribonucleotide reductase R2 through its 3′-UTR (21). In the case of Cited2, 3′-UTR does not respond to TGF-β-mediated regulation, when linked downstream of CMV-driven luciferase reporter (Fig. 3D). Even though typical AU-rich destabilization sequences are not observed in 3′-UTR of Cited2, we detect Mos polyadenylation-response element (Mos-PRE) in 3′-UTR. Mos polyadenylation sites in the 3′-UTR of Mos mRNA are responsible for cytoplasmic polyadenylation and translational activation of Mos mRNA during progestosterone-stimulated Xenopus oocyte maturation (47). Progesterone induces Cited2 mRNA expression in the mouse uterus (48). It will be interesting to test whether the Mos-PRE in 3′-UTR of Cited2 is necessary for progesterone-induced mRNA expression of Cited2.

To further elucidate the mechanism of TGF-β-mediated down-regulation of Cited2, we analyzed the mRNA expression of the recombiant Cited2 gene in MDA-MB-231 cells. Consistent with the notion that the regulation occurs at the post-transcriptional level, we observed correct regulation of exogenous Cited2 mRNA driven by a viral promoter, murine leukemia virus-long terminal repeat, suggesting that the protein coding sequence is sufficient to confer Cited2 down-regulation by TGF-β. To our knowledge, this is the first demonstration that the mRNA stability of a TGF-β down-regulated gene is controlled by its coding sequence. Coding sequences are necessary for the mRNA stability of IL-11, c-Myc, c-Fos, elastin, luteinizing hormone receptor, albumin, and yeast MAT1α (36, 37, 40, 49–52). The stability of these and many other labile mRNAs has been shown to be coupled to translation (40, 49, 50, 53), and polysome-associated endonucleases may be involved in this process (50, 54, 55). We have shown previously that phorbol ester-induced IL-11 mRNA stabilization is in part through the IL-11 coding sequence (36, 37). Wisdom and Lee (40) reported that c-Myc mRNA is down-regulated during myogenic differentiation, and the down-regulation is mediated by accelerated mRNA decay. Stable expression of exogenous c-Myc driven by metallothionein promoter is also down-regulated as the endogenous c-Myc mRNA by a differentiation agent, hexamethylene bisacetamide (HMBA), in mouse erythroleukemia cells (56). A C-terminal 249-nucleotide coding region instability determinant (CRD) was identified to be responsible for the c-Myc mRNA instability (57). A CRD-binding protein (CRD-BP) binds to this region and appears to protect the c-Myc mRNA from endonuclease cleavage (57). It is proposed that during myoblast differentiation, RNA binding activity of CRD-BP is decreased, which in turn accelerates the degradation rate of c-Myc mRNA (58). Translation of c-Myc mRNA is also necessary for its down-regulation during myogenesis (40, 59). Translation pausing occurs in the CRD of c-Myc mRNA because of rare codons, such as arginine (CGA) and threonine (ACA), and generates a ribosome-deficient region downstream of the pausing site (58). The ribosome-deficient region of c-Myc is exposed to endonuclease attack unless it is shielded by the CRD-BP (58). We have observed increased stability of Cited2 mRNA once the C terminus of Cited2 coding region was removed (Fig. 5, D–G), suggesting that the C terminus is essential for the down-regulation and may function as a CRD of Cited2. Whether there are trans factors binding to the CRD of Cited2 to protect the degradation of Cited2 transcript and whether Cited2 down-regulation involves translation pausing in the Cited2 coding sequence requires further study.

By overexpression of Smad7, an inhibitory Smad, in MDA-MB-231 cells, we showed that down-regulation of Cited2 by TGF-β is attenuated (Fig. 2, A and B). Consistent with this result, when Smad4 was knocked down in MDA-MB-231 cells, the TGF-β-mediated effect on Cited2
down-regulation was attenuated (Fig. 2C), supporting that the Smad pathway is involved in the regulation of Cited2 expression. Smad2 and Smad3 may work independently or in cooperation with each other to mediate gene expression. In this work, we further reveal that knockdown of both Smad2 and Smad3 effectively attenuated TGF-β-mediated down-regulation of Cited2, suggesting that common Smad, Smad4, may work with either Smad2 or Smad3 for the down-regulation of Cited2. BMP2 also executes signaling through Smad4, but BMP2 activates different receptor Smads, Smad1/5/8. Unlike TGF-β, BMP2 does not down-regulate Cited2, suggesting that down-regulation of Cited2 is mediated by specific receptor Smads. Because individual receptor Smads interact with different transcription factors and coactivators (60), it is possible that Smad2/3 interacting proteins may determine the expression of Cited2. Although Smad pathway was inhibited through overexpression of Smad7 or si-Smads, we observed increased basal expression of Cited2. Because MDA-MB-231 is a cell line with high TGF-β-autocrine activity (61, 62), it is likely that increased basal expression of Cited2 is because of the attenuation of TGF-β autocrine by blockade of the Smad pathway through Smad7 and si-Smads. TGF-β also induces the stabilization of elastin mRNA in lung fibroblasts through the Smad pathway and elastin coding region, although the detailed mechanism involved is not clear (22, 50). It is possible that the Smad pathway may change Cited2 mRNA stability by affecting the activity or expression of mRNA-binding proteins, thereby increasing the turnover rate of the Cited2 transcript.

It is interesting that Cited1, another member of the Cited family, is also down-regulated by TGF-β in B16-F1 melanoma (16). The mechanism of Cited1 down-regulation by TGF-β is unclear. In this study, we found that Cited2 is regulated by TGF-β through post-transcriptional control in MDA-MB-231 cells. Members of the Cited family share the C-terminal conserved region. Deleting the C-terminal conserved region of Cited2 resulted in the loss of Cited2 down-regulation by TGF-β, suggesting that TGF-β may down-regulate Cited1 by a similar mechanism as Cited2. TGF-β modulates downstream signaling by regulating expression of many transcription factors such as junB, ATF3, and Id1 (43). Both Cited1 and Cited2 interact with CBP/p300 and function as transcriptional coactivators. Cited1 interacts with Smad4 and enhances TGF-β-mediated transcription (16), suggesting that members of the Cited family may play roles in TGF-β signaling. Recently, we found that Cited2 enhances TGF-β-mediated MMP9 expression in MDA-MB-231 cells and promotes tumor cell migration in Matrigel assays (29). In addition, Cited2 enhances NGAL(24p3) expression, which is further confirmed by the fact that knockdown of Cited2 in MDA-MB-231 cells decreases NGAL(24p3) expression (Fig. 7). NGAL(24p3) forms complexes with MMP9 and protects MMP9 from autodegradation (44), which in turn facilitates breast cancer progression (64). In addition to MDA-MB-231 breast cancer cells, Cited2 is highly expressed in Ewing sarcomas and Engelbreth-Holm-Swarm tumors (65, 66). We have shown that Cited2 is a transforming gene and overexpression of Cited2 in Rat1 cells leads to tumor formation in nude mice (1). Because TGF-β plays important roles during tumorigenesis (67), our current hypothesis is that by down-regulation of Cited2, TGF-β modulates MMP9 (29) and NGAL(24p3) levels, which may further create a specific and temporal control on downstream events during tumor progression.

In conclusion, we demonstrate that Cited2 is down-regulated by TGF-β in MDA-MB-231 cells through the Smad pathway. The down-regulation of Cited2 by TGF-β is post-transcriptional in part through the accelerated decay of Cited2 mRNA. TGF-β-regulated Cited2 mRNA stability is coupled to its translation. We reveal that the C-terminal Cited2 coding sequence is necessary for TGF-β-mediated down-regulation. Future studies will attempt to localize and define the elements in the coding sequence targeting Cited2 mRNA down-regulation and to identify trans factors that mediate its down-regulation under TGF-β stimulation.

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TGF-β-mediated Down-regulation of Cited2

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