Modulation of Transforming Growth Factor β (TGFβ)/Smad Transcriptional Responses through Targeted Degradation of TGFβ-inducible Early Gene-1 by Human Seven in Absentia Homologue*

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Transforming growth factor β (TGFβ)-inducible early gene-1 (TIEG1) is a Krüppel-like transcription factor that is rapidly induced upon TGFβ treatment. TIEG1 promotes TGFβ/Smad signaling by down-regulating negative feedback through the inhibitory Smad7. In this report, we describe the identification of an E3 ubiquitin ligase, Seven in Absentia homologue-1 (SIAH1), as a TIEG1-interacting protein. We show that TIEG1 and SIAH1 interact through an amino-terminal domain of TIEG1. Co-expression of SIAH1 results in proteasomal degradation of TIEG1 but not of the related factor TIEG2. Importantly, co-expression of SIAH1 completely reverses repression of Smad7 promoter activity by TIEG1. Furthermore, overexpression of a dominant negative SIAH1 stabilizes TIEG1 and synergizes with TIEG1 to enhance TGFβ/Smad-dependent transcriptional activation. These findings suggest a novel mechanism whereby the ability of TGFβ to modulate gene transcription may be regulated by proteasomal degradation of the downstream effector TIEG1 through the SIAH pathway. In this manner, turnover of TIEG1 may serve to limit the duration and/or magnitude of TGFβ responses.

Transforming growth factor-β (TGFβ)1-inducible early gene-1 (TIEG1) is a member of the Krüppel-like family of zinc finger transcription factors and is induced by several members of the TGFβ superfamily including TGFβ and bone morphogenetic protein-2 (BMP-2) (1, 2). The carboxyl-terminal three zinc finger DNA binding domain allows TIEG1 to directly associate with GC-rich sequences of DNA in target gene promoters (3–5). The three zinc fingers are highly conserved between TIEG1 and the related transcription factor TIEG2, suggesting that they may regulate the same sequences in target gene promoters (6). However, outside of the DNA binding domain, TIEG1 and TIEG2 bear little sequence homology. Yet both factors are negative regulators of gene transcription and contain an α-helical repression domain (3, 5, 7, 8). This domain is capable of repressing gene transcription when fused to a heterologous DNA binding domain and can directly interact with the transcriptional co-repressor mSin3A (7, 8). Overexpression of TIEG1 mimics TGFβ action in several cell types by modulating differentiation markers, decreasing proliferation, and inducing apoptosis (9–12). Accumulating in vitro and clinical data in the literature suggests that TIEG1 serves a tumor suppressor role because overexpression reduces proliferation and induces apoptosis (9–12). Further support for this role arose from our studies demonstrating a decrease in TIEG1 protein levels that coincides with the increasing stages of cancer in breast tumor biopsies (13).

TGFβ is a pleiotropic cytokine known to regulate many cellular processes including differentiation, proliferation, apoptosis, and migration whereas BMPs have been shown to play critical roles in development and bone formation. TGFβ or BMP binding to its cognate transmembrane type II receptor initiates formation and activation of a heteromeric complex with the corresponding type I receptor (14). Upon activation, the type I receptor initiates intracellular signaling by phosphorylation of the receptor-regulated Smad (R-Smad) proteins. Smad2 and -3 function in TGFβ signaling, whereas Smad1, -5, and -8 function in BMP signaling (15, 16). Phosphorylated R-Smads complex with the common mediator Smad4, translocate to the nucleus, and modulate target gene transcription. Negative regulation of the Smad pathway occurs through the inhibitory Smads, Smad6 and -7. Smad7 blocks both TGFβ and BMP signaling by binding to the type I receptor and preventing phosphorylation of the R-Smads as well as increasing turnover of the receptor (17–21). Smad6 specifically blocks BMP signaling by binding the TGFβ-activated kinase-1 and by binding Smad4, thereby preventing Smad4 heteromerization with Smad1, -5, and -8 (18, 22). TIEG1 was recently shown to play a unique role in TGFβ/Smad signaling by down-regulating negative feedback through Smad7. By repressing Smad7 gene transcription, TIEG1 is able to enhance transcription of important TGFβ-regulated genes such as the cyclin-dependent protein kinase inhibitor p21 and the plasminogen activator inhibitor-1 (PAI-1) (5). We have also observed that TIEG1 is able to repress Smad6 pro-
moter activity and may therefore serve an important role in BMP signaling as well.\(^2\)

Regulation of protein stability through the ubiquitin-proteasome pathway is now being recognized as a major mechanism of regulating a diverse array of cellular processes (23). Ubiquitination is carried out in a three-step process requiring three different proteins referred to as E1, E2, and E3 (23). The ubiquitin-activating enzyme (E1) undergoes a covalent intermediate in which the carboxyl terminus of ubiquitin forms a high energy thioester bond in an ATP-dependent process with an active cysteine residue in the E1 molecule. Another thioester intermediate is formed when ubiquitin is transferred from E1 to the E2 protein, also referred to as a ubiquitin-conjugating enzyme. Specificity of substrate recognition is provided by the E3 ubiquitin ligase, which facilitates the transfer of ubiquitin to the target protein (23).

The Seven in Absentia (Sina) protein was identified in a screen for mutants defective in the development of the R7 photoreceptor cell in Drosophila melanogaster (24). It was subsequently shown to function in signaling downstream of the receptor tyrosine kinase Sevenless by targeting a transcriptional repressor, Tramtrack, for degradation by the ubiquitin-proteasome pathway (25). Degradation of Tramtrack will then result in derepression of target genes such as the pair-rule genes even-skipped and fushi tarazu (26). The human homologues of Sina, SIAH1 and SIAH2, are able to target both nuclear and cytoplasmic proteins for proteasomal degradation (27–33).

We demonstrate that the stability of TIEG1 protein is regulated by interaction with the human homologue of Seven in Absentia, SIAH1. Thus we propose a pathway in mammalian cells that closely parallels the Drosophila pathway in which degradation of a transcriptional repressor (Tramtrack or TIEG1) by a ubiquitin ligase (Sina or SIAH) results in derepression of genes encoding important cellular proteins (even-skipped or Smad7) (27). This novel interaction suggests a role for the regulation of TIEG1 protein stability in modulating the activity of the TGFβ signal transduction pathway.

**MATERIALS AND METHODS**

**Yeast Two-hybrid**—The bait plasmid was constructed by cloning the region containing amino acids 1–350 of TIEG1 (1) into pAS2-1 (CLONTECH, Palo Alto, CA) by PCR. This plasmid was transformed into the MATa yeast strain AH109 using the Yeastmaker Transformation System (CLONTECH). The yeast two-hybrid screen was performed using a pretransformed skeletal muscle library (CLONTECH) and interacting clones identified by the ability to grow on minimal SD agar medium lacking tryptophan, leucine, histidine, and adenine (CLONTECH). Prey plasmids were recovered and retransformed into AH109 with the bait or the GAL4 DNA binding domain alone to verify interactions by β-galactosidase filter lift assays. The identity of clones containing β-galactosidase was determined by DNA sequencing.

**Plasmid Constructs**—The amino-terminal FLAG epitope-tagged TIEG1 and TIE2 expression vectors were constructed by amplifying the respective open reading frames by PCR with primers that add EcoRI and BamHI or EcoRI and XbaI restriction sites at the 5’- and 3’-ends, respectively. The resulting fragment was cloned into pCDNA4/TO (Invitrogen) behind a FLAG epitope tag. The Renilla luciferase plasmid (pRL-CMV) was purchased from Promega (Madison, WI). The SIAH1 expression vector was constructed by cloning the entire SIAH1 coding region into pCDNA4/TO. FLAG-tagged dominant negative SIAH1 (dn-SIAH1), which contains a cysteine to serine mutation at residue 72, was constructed by PCR and cloned into pCDNA4/TO. Sequence integrity was verified by DNA sequencing.

**In Vitro Binding Assays**—GST fusion proteins were generated by cloning portions of TIEG1 into pGEX-2T-6His-PL2. Fusion proteins were purified as previously described (34). Recombinant \(^35\)S-labeled SIAH1 was produced from pGIBR-T7-SIAH1 using the \(T7\) et in vitro transcription and translation system (Promega). GST fusion proteins were immobilized on glutathione-Sepharose 4B (Amersham Biosciences) and incubated with \(^35\)S-labeled SIAH1 at 4°C for 2h in 50 mM NaH2PO\(_4\) (pH 7.2), 100 mM NaCl, and 0.05% (v/v) Triton X-100 with gentle mixing. The mixtures were then centrifuged, and pellets were washed extensively in the same buffer. Bound proteins were eluted by boiling in Laemmli buffer and separated by SDS-PAGE using precast 10% (v/v) Tris-HCl polyacrylamide gels (Bio-Rad). Radiolabeled SIAH1 was visualized using a Storm 840 PhosphorImager (Amersham Biosciences).

**Cell Culture and Cell Transfection**—293C12 mouse myoblast cells were obtained from ATCC (Manassas, VA) and grown in Dulbecco’s modified Eagle’s medium/F12 (1:1) (Sigma) containing 10% (v/v) fetal bovine serum (Bio Whittaker, Walkersville, MD) and 1× antibiotic-antimycotic solution (Invitrogen). Cells were seeded in 12-well or 4-cm plates and transfected at 50% confluence with various combinations of plasmid DNA using LipofectAMINE Plus (Invitrogen) according to the manufacturer’s directions. All transfections included a plasmid containing Renilla luciferase driven from the cytomegalovirus promoter (pRL-CMV) as an internal transfection control.

**Western Blotting**—Cells extracts were harvested in radioimmunoprecipitation assay buffer (phosphate-buffered saline, 1% (w/v) Nonidet P-40, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS) containing 100 μg/ml phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin, 10 μg/ml leupeptin, and 100 μM sodium orthovanadate. Proteins were separated on a 50 μl SDS-10% (w/v) polyacrylamide gel and blotted onto Protran nitrocellulose membranes (Schleicher & Schuell). TIEG1 protein was detected with a rabbit polyclonal antibody, TIEG-228, raised to a synthetic peptide corresponding to amino acids 133–154 of the human TIEG1 protein. Actin and FLAG epitope-tagged proteins were detected with anti-actin AC-40 and anti-FLAG M2 antibodies, respectively (Sigma). Primary antibodies were detected by enhanced chemiluminescence (Amersham Biosciences) using horseradish peroxidase-conjugated anti-rabbit or anti-mouse secondary antibodies (Sigma).

**Luciferase Promoter Reporter Assays**—Cell extracts were harvested 48 h after transfection using 300 μl of passive lysis buffer. Luciferase assays were performed using the dual luciferase reporter assay system (Promega) and read on a Turner TD-20/20 luminometer. To correct for differences in transfection efficiency, firefly luciferase units were normalized relative to Renilla luciferase units of the same sample. Corrected luciferase values were then expressed as a ratio (-fold induction) relative to the vector control transfected cells.

**RESULTS AND DISCUSSION**

**Identification of SIAH1 as a TIEG1-interacting Protein**—To identify proteins that interact with TIEG1, we utilized the yeast two-hybrid system. Because TIEG1 is a member of the Kruülp-like factor family of three zinc finger transcription factors, which bear a high degree of homology to one another in the carboxyl-terminal DNA binding domain, we excluded this domain by utilizing a bait construct containing only the first 350 amino acids of TIEG1 (Fig. 1A) to screen a skeletal muscle library. This region contains several potential SH3 binding domains as well as three distinct repression domains (1, 7). Four positive clones, which grew on selective media and were positive for β-galactosidase expression, were identified from 1.6 × 10\(^7\) clones that were screened. All four clones contained inserts identified as the human homologues of the D. melanogaster seven in absentia gene. Three of the clones contained seven in absentia homologue (SIAH)-2 whereas the other contained SIAH1. SIAH1 and SIAH2 share 87% amino acid identity to one another and 76 and 68% to Sina, respectively (35). Because these two proteins appear to be functionally redundant and similarly recognize substrate proteins (27) and because SIAH1 has been extensively characterized, we focused primarily upon the interaction between TIEG1 and SIAH1. As shown by growth on selective medium, both full-length TIEG1 as well as TIEG1\(^{1-350}\) specifically interact with SIAH1, whereas TIEG1 did not do so with a control protein, the estrogen receptor-α (Fig. 1B). Furthermore, as previously reported, SIAH1 also interacts with itself (25, 36).

As shown in Fig. 1C, using in vitro binding assays TIEG1 appears to interact with SIAH1 through the amino-terminal
210 amino acids because all three constructs containing this region (TIEG11-480, TIEG11-350, and TIEG11-210) were able to pull SIAH1 down. However, a stronger interaction between TIEG1 and SIAH1 is observed when the entire amino-terminal domain remains intact (TIEG11-350; Fig. 1C). This suggests that some aspect of the TIEG1 tertiary structure is necessary for optimal interaction between TIEG1 and SIAH1 or that amino acids 211–350 augment binding.

**SIAH Targets TIEG1 for Proteasomal Degradation**—Based on previous data showing that both Sina and SIAH1 function as E3 ubiquitin ligases and target certain proteins for proteasomal degradation, we hypothesized that the interaction with SIAH1 may increase turnover of TIEG1 protein levels. Indeed, when a SIAH1 expression vector was co-transfected into C2C12 cells with a TIEG1 expression vector, a dramatic decrease in TIEG1 protein levels was observed. In fact, only small amounts of SIAH1 expression vector (75 ng) were necessary to decrease TIEG1 protein to undetectable levels, and even a very small amount (5 ng) had a visible effect (Fig. 2A). As a control, actin protein levels were unaffected. Thus the effect of SIAH1 expression on TIEG1 protein levels is considerably more dramatic than that observed for other SIAH1 target proteins such as β-catenin, deleted in colorectal cancer (DCC), and synaptophysin, in which there is only a partial decrease in protein levels even with high amounts of SIAH1 expression (27, 32, 37). Conversely, expression of a dominant negative SIAH1 (dn-
SIAH1), in which the RING finger structure required for E3 ligase activity has been obstructed, appears to stabilize TIEG1 protein levels (Fig. 2A). In addition, the proteasome inhibitor MG132 completely blocks the decrease in TIEG1 protein levels upon SIAH1 co-expression, suggesting that the decrease in TIEG1 protein levels is proteasome-dependent (Fig. 2B). Furthermore, degradation of TIEG1 by SIAH1 is specific because SIAH1 has no effect on TIEG2 protein levels (Fig. 2B).

**SIAH1 Reverses Repression of the Smad7 Promoter by TIEG1**—Regulation of gene expression through targeted degradation of important transcription factors is a common theme in transcriptional control. For example, the protein levels and activities of p53, β-catenin, and NFκB are all regulated by targeted degradation of specific proteins through the ubiquitin-proteasome pathway (38–41). Therefore, based on the observation that SIAH1 targets TIEG1 for proteasomal degradation, we reasoned that SIAH1 may also influence the regulation of TIEG1 target genes. Simply stated, increased SIAH1 activity might decrease TIEG levels and reverse any TIEG protein effects on gene expression, whereas inhibition of SIAH1 activity may enhance these effects. Although we recently demonstrated that TIEG1 represses Smad7 expression by binding to a GC-rich sequence in the proximal Smad7 promoter (5), we now find that overexpression of SIAH1 by itself has very little effect on Smad7 promoter activity (Fig. 3A). This observation may be explained by the fact that, in the absence of growth factor treatment, TIEG1 is expressed at levels too low for significant biological activity (1, 2, 9). Thus a subsequent reduction of TIEG protein levels by SIAH1 would be predicted to have no observable effect on the regulation of TIEG target genes, whereas an increase in endogenous TIEG protein levels, either through the inhibition of SIAH activity or overexpression of TIEG, should have a noticeable effect. Indeed, overexpression of dn-SIAH1 or TIEG1 alone decreases Smad7 promoter activity (Fig. 3A).

The effect of SIAH1 is more evident when TIEG1 is co-expressed. As shown in Fig. 3B, TIEG1 dramatically represses Smad7 promoter activity. However, co-expression of SIAH1 restores normal promoter activity. The reversal of Smad7 repression by SIAH1 is also specific for TIEG1 because TIEG2 also represses Smad7 promoter activity (Fig. 3B), but SIAH1 does not abrogate this repression.

**SIAH1 Influences TGFβ/Smad Signaling**—The ubiquitin-proteasome pathway plays an important role in regulating TGFβ signal transduction through the Smad proteins. Most notably, activation of the TGFβ/Smad pathway stimulates an interaction between Smad2 and the Smad ubiquitination regulatory factor-2 (Smurf-2), an E3 ubiquitin ligase, and results in the ubiquitination and subsequent degradation of Smad2 (42, 43). Similarly, Smad3 is degraded through interaction with another ubiquitin ligase complex, the anaphase promoting complex, following ligand stimulation (44). In cancer cells, expression of oncogenic Ras or the presence of specific destabilizing mutations promotes ubiquitin-proteasome-dependent turnover of Smad4, rendering cells insensitive to the effects of TGFβ (45–47). Thus the ubiquitin-proteasome pathway plays an important role in regulating the TGFβ/Smad pathway.

Because repression of Smad7 promoter activity by TIEG1
enhances TGFβ/Smad signaling by relieving negative feedback imposed by Smad7, it is likely that modulation of TIEG1 protein levels would also have an effect on the entire Smad pathway. Therefore we hypothesized that SIAH1 or dn-SIAH1 overexpression may influence Smad signaling by regulating TIEG1 stability. This was tested using a TGFβ-responsive reporter construct (CAGA_{12}-MLP-Luc) and a constitutively active type I TGFβ receptor (ALK5TD) along with co-expression of TIEG1 and SIAH1 or dn-SIAH1. As shown in Fig. 4 and similar to the effects observed with the Smad7 promoter, expression of wild-type SIAH1 has virtually no effect on CAGA_{12}-MLP-Luc reporter induction by itself and slightly reverses the enhancement observed with TIEG1 co-expression. However, dn-SIAH1 overexpression enhances TGFβ/Smad signaling to a similar degree as TIEG1 overexpression by increasing ALK5TD-induced transcription—2-fold compared with vector transfected cells. Interestingly, co-expression of dn-SIAH1 and TIEG1 appears to synergistically enhance CAGA_{12}-MLP induction to about 5.5-fold compared with vector transfected cells. This effect is probably because of stabilization of TIEG1 protein.

These data suggest that activation of the TGFβ/Smad pathway may be regulated through the ubiquitin-proteasome pathway at an additional level by the regulation of the stability of the TGFβ-inducible early protein through SIAH1. By controlling TIEG1 protein levels a cell has an additional mechanism to control the amplitude or duration of TGFβ signaling.

This also provides a mechanism that cancer cells may employ to evade cell cycle regulation by TGFβ. Mutations in the TGFβ pathway have been observed in certain types of cancer including pancreatic and colorectal cancers (48). Most of these mutations result in a complete blockage of TGFβ signaling through the Smad pathway and are believed to relieve the cancer cells from the growth inhibitory effects of TGFβ. However, in many cases there appears to be subtle perturbations in the activity of TGFβ signaling that are not because of mutations in the known signaling components (48). The effects of these subtle changes often increase the aggressiveness and/or metastatic potential of a tumor. Interestingly a decrease in TIEG1 protein levels correlates with the histological stage of breast cancer (13). However, it is currently unknown whether the mRNA levels also correlate. It is possible that the decrease in TIEG1 protein levels may be because of post-translational regulation of TIEG1 protein levels, possibly by the SIAH proteins or another, as yet, unidentified ubiquitin ligase. Investigations into the expression of the SIAH proteins and the regulation of the SIAH genes may provide insights into the regulation of the TGFβ/Smad signaling pathway and may hint at a new area of exploration in cancer research.

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