TSG101 Protein Steady-State Level Is Regulated Posttranslationally by an Evolutionarily Conserved COOH-Terminal Sequence

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ABSTRACT

Antisense inactivation of the tsg101 tumor susceptibility gene in murine NIH3T3 fibroblasts leads to neoplastic transformation and tumorigenesis, which are reversed by restoration of tsg101 activity. tsg101 deficiency is associated with a series of mitosis-related abnormalities, whereas overexpression of TSG101 can also result in neoplastic transformation and the perturbation of cell cycling. Together, these observations imply that TSG101 production outside of a narrow range can lead to abnormal cell growth. We report here that the TSG101 protein is maintained at an almost constant steady-state level in cultured murine and human cells and that this occurs through a posttranslational process involving TSG101 protein degradation. Sustained overproduction of TSG101 from chromosomally inserted adventitious constructs results in compensatory down-regulation of endogenous TSG101 and replacement of the native protein by the adventitious one. Using deletion mutants of TSG101, we mapped the region responsible for autoregulation of the TSG101 steady-state level to an evolutionarily conserved sequence, here termed the "steadiness box," located near TSG101’s COOH-terminal end. Our results suggest a model in which the biological effects of TSG101 are modulated either by self-promoted proteolysis or participation with other cellular protein(s) in a proteolytic feedback-control loop.

INTRODUCTION

Functional inactivation of the tsg101 tumor susceptibility gene by antisense RNA in murine NIH3T3 fibroblasts leads to colony formation in 0.5% agar, focus formation in monolayer cultures, and the ability to form metastatic tumors in athymic nude mice (1). Restoration of tsg101 activity reverses these features of neoplastic transformation as well as the nuclear, microtubule, and mitotic spindle abnormalities observed in TSG101-deficient cells (1, 2). Initial PCR-based findings of frequent intragenic DNA deletions within TSG101 in human breast cancers (3) have not been reproducible (4), and overexpression of TSG101 also has been associated with GBM (19). This implies that either elevation or diminution of the TSG101 steady-state level can lead directly or indirectly to abnormal cell growth. A corollary of this statement is that TSG101 expression normally may be stringently controlled. We report here investigations that address the mechanism of regulation of TSG101 protein level. We show that intracellular TSG101 protein is in fact maintained within a narrow range in cultured cell populations by a posttranslational mechanism that modulates TSG101 protein degradation and prevents its accumulation in cells overexpressing TSG101 mRNA. We further show that regulation of the steady-state level of intracellular TSG101 protein is mediated by an evolutionarily conserved sequence (termed the SB box) located near its COOH terminus.

MATERIALS AND METHODS

Cell Culture and Transfection. Human Ecr293 and mouse Ecr3T3 cell lines (Invitrogen, San Diego, CA), which were used to study inducible expression of TSG101, can express adventitious genes under control of a modified promoter containing Drosophila ecdysone-responsive DNA elements. These cell lines and their derivatives were grown in DMEM supplemented with 10% fetal bovine serum (Life Technologies, Inc., Gaithersburg, MD) and appropriate antibiotics. LipofectAMINE (Life Technologies, Inc.) was used to transfected linearized DNA constructs into the cells according to procedures recommended by the manufacturer. Stable transfectants were selected and expanded in media containing G418 at 600–800 µg/ml. To induce expression of the adventitious proteins, cells at 40–50% confluence were treated with analogues (650) 725-1536; E-mail: sncohen@stanford.edu.

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4 The abbreviations used are: SB, steadiness box; AA, amino acid; MA, muristerone A; PA, ponasterone A; UTR, untranslated region.
REGULATION OF TSG101 PROTEIN LEVEL

**Antibodies.** Antisera against the full-length murine TSG101 protein or the COOH-terminal 20 AAs were raised in rabbits, and the antibodies were purified by affinity chromatography as described previously (2). Other antibodies used in this research included the M2 mouse monoclonal antibody (Kodak, New Haven, CT) for detection of the Flag peptide and the antibody against α-tubulin (Sigma, St. Louis, MO) for normalization of loading.

**Gel Electrophoresis and Immunoblotting.** Proteins in cell lysates were separated by SDS-PAGE, electrotransferred to nitrocellulose filters, and analyzed by enhanced chemiluminescence (Amersham, Buckinghamshire, United Kingdom).

**Protein Labeling and Immunoprecipitation.** Cells grown to about 50% confluence were washed and incubated for 1 h in methionine-free DMEM (Life Technologies, Inc.), followed by labeling of proteins in 150 μCi/ml [35]methionine for the designated time. In pulse-chase experiments, the labeling time was 1 h. For immunoprecipitations, labeled cells were washed in PBS twice before being lysed in 2 ml of cold radioimmunoprecipitation assay buffer [50 mM Tris-Cl (pH 7.5), 0.15 M NaCl, 1% Triton X-100, 0.5% deoxycholate, 0.1% SDS, and protease inhibitors]. The lysates were centrifuged at 4000 rpm for 5 min, and supernatants were either stored at −80°C or used directly. Individual samples containing equal volumes of labeled extracts were incubated for 1 h with 2 μg of nonspecific rabbit IgG and 30 μl of protein A-agarose beads (Pharmacia, Uppsala, Sweden) on a rocking rotary shaker in a cold room. The supernatant was then incubated with an excess amount of specific antibody and 30 μl of protein A-agarose beads overnight. The beads were washed four times with radioimmunoprecipitation assay buffer, and bound proteins were dissolved by boiling in the loading buffer, separated on SDS-PAGE gels, and analyzed by autoradiography. Protein concentrations were estimated by serial dilution of the protein samples before loading onto gels and by spectroscopic analysis of band intensities on X-ray films.

**Northern Blot Analysis.** Total RNAs were isolated from cells by RNA STAT-60 (Tel-Test, Friendswood, TX), separated in agarose gels under denaturing conditions, and transferred using capillaries to Hybond-N nylon membranes (Amersham Life Sciences). Hybridization and washing were performed under stringent conditions as described by Sambrook et al. (20).

**RESULTS**

Overexpression of Adventitious TSG101 Protein Leads to a Decrease in Endogenous TSG101. In experiments initially aimed at overproducing TSG101 protein, we placed mouse and human TSG101 cDNAs containing the full-length coding sequence under the control of a promoter inducible by MA or PA and introduced these constructs by transfection into human Ecr293 cells to generate stable cell lines. However, after induction, we observed little or no increase in expression of the adventitious protein under different inducer concentrations. Cultures were induced for 24 h using different concentrations of MA. A, expression of the adventitious protein after removal of the inducer. Cells were first cultured in medium containing 1 μM MA for 24 h and then cultured in medium lacking inducer for up to 48 h. They were harvested at the time points for immunoblot analysis. B, detection of the adventitious protein in subculture passages continuously under induction. One-sixth of the previous cell culture was inoculated for the next passage in each subculture. All subcultures were grown either in the absence of inducer (0 μM MA) or in the continuous presence of inducer (1 μM MA) until harvested. P1–P5, passages 1–5.

**Fig. 2. Inverse correlation between the endogenous and adventitious TSG101 proteins.** A representative stable cell line, human Ecr293 transfected by the Flag-tagged mouse cDNA construct, was induced to express the adventitious Flag-tagged mouse TSG101 protein (Flag-TSG101). Total protein from 8 × 10^6 cells was loaded in each lane. Antibody against the full-length TSG101 protein was used for immunoblot analysis, whereas antibody against α-tubulin was used as an internal control to normalize loading. A, time course of induced expression of the adventitious protein. Cells at 40% confluence were induced by adding 1 μM MA to the medium and harvested at the times shown. B, expression of the adventitious protein under different inducer concentrations. Cultures were induced for 24 h using different concentrations of MA. C, expression of the adventitious protein after removal of the inducer. Cells were first cultured in medium containing 1 μM MA for 24 h and then cultured in medium lacking inducer for up to 48 h. They were harvested at the time points for immunoblot analysis. D, detection of the adventitious protein in subculture passages continuously under induction. One-sixth of the previous cell culture was inoculated for the next passage in each subculture. All subcultures were grown either in the absence of inducer (0 μM MA) or in the continuous presence of inducer (1 μM MA) until harvested. P1–P5, passages 1–5.
in total TSG101 protein from either construct by Western blot analysis of whole cell extracts using antibody to full-length TSG101 as probe (Fig. 1A). To determine whether TSG101 protein actually was being expressed from adventitious constructs and to distinguish induced TSG101 from the endogenous protein, we attached a nucleotide segment encoding an 8-aa Flag sequence plus three additional spacer AAs (see “Materials and Methods”) to the 3’ ends of human and murine TSG101 protein coding sequences and generated Ecr293 (human) and Ecr3T3 (murine) stable transfectants containing these constructs. After the addition of MA to these cultures, Western blot analysis of cell extracts using antibodies made against either full-length TSG101 or its COOH-terminal end detected human and murine cDNA-encoded protein bands that migrated more slowly in gels (calculated molecular weights of 49,000 and 50,000, respectively, in both murine and human cells) than the respective endogenous TSG101 proteins, which migrate near $M_r$ 46,000 (Fig. 1, B–D). Whereas the positions of these bands were higher than expected from addition of the 11-aa Flag peptide and spacer to full-length TSG101 proteins, immunoprecipitations from Ecr293 cells by antibody against the Flag peptide (Fig. 1E, right) confirmed that the slowly migrating species made in these cells is Flag-tagged TSG101 protein derived from the induced adventitious construct (Fig. 1E, left) rather than a modified form of endogenous TSG101.

Additional Western blot analysis of Flag-tagged TSG101 in gels showed that MA-induced expression of adventitious TSG101 protein resulted in concomitant down-regulation of the resident protein, leading to replacement of endogenous TSG101 by the adventitious protein in both human Ecr293 cells and mouse Ecr3T3 cells (Fig. 2). Because similar results were observed in these cell lines during expression of adventitious mouse and human TSG101 cDNAs, the cDNAs and cell lines were used interchangeably in subsequent experiments. As seen in Fig. 2, the extent of replacement of the endogenous protein varied with the period of induced expression of the adventitious Flag-tagged protein (Fig. 2A) and the concentration of inducer (Fig. 2B). In the absence of inducer, little or no adventitious protein was expressed, indicating effective control of the regulated promoter. Adventitious TSG101 protein was first detected in elevated amounts 4 h after induction, reached its maximum of twice the native level of endogenous protein at 8 h, and was maintained at this level throughout the experiment as long as inducer was present (Fig. 2A). After 8 h of induced expression of adventitious TSG101 by 1 $\mu$M MA, endogenous

![Fig. 3. Northern blot analysis of induced TSG101 transcripts. Left, total TSG101 transcripts, including the adventitious and endogenous transcripts in Ecr293 cells, were analyzed at different concentrations of MA. The probe used to detect the sum of mouse and human TSG101 transcripts was a mixture of both mouse and human full-length TSG101 cDNAs in equal amount. Middle, the endogenous transcript alone was analyzed in the same RNA samples using the endogenous transcript-specific 3′-UTR of human TSG101 cDNA as probe. Right, ethidium bromide-stained agarose gels served as loading controls for RNA samples.](image)

TSG101 began to decrease, and at 24 h, it was present in this experiment at 20% of the initial level (Fig. 2A); the precise extent of reduction of endogenous TSG101 varied slightly among different experiments. At a MA concentration of 10 $\mu$M, adventitious TSG101 protein was present at more than 10 times the amount of endogenous protein after 24 h of induction (Fig. 2B).

An inverse correlation between the intracellular levels of endogenous and adventitious TSG101 protein was also observed when expression of the adventitious protein was turned off by transfer of MA-induced cells to medium lacking inducer (Fig. 2C). As Flag-tagged adventitious TSG101 decayed, endogenous TSG101 gradually returned to its normal steady-state level. The ratio of Flag-tagged adventitious TSG101 protein:endogenous native protein remained unchanged during subculturing of continuously induced transfected cell lines for more than 30 doublings (Fig. 2D), indicating that the observed replacement of endogenous protein with adventitious protein did not result from selective growth of induced cells. Interestingly, the observed 1.5- to 2-fold increase in total (i.e., endogenous plus adventitious) TSG101 protein at the time of maximum induction was associated with cell clumping and a more rounded cell morphology compared with inducer-treated cells that did not overexpress TSG101 protein.
Down-Regulation of TSG101 Protein Is Posttranslational. In contrast to the observations made for TSG101 protein, Northern blot analysis showed that TSG101-specific mRNA increased 6- to 10-fold 24 h after the addition of 1 or 10 μM MA to Ecr293 cells containing the Flag-tagged murine TSG101 construct (Fig. 3, left). During this time, endogenous (human) TSG101 transcripts, which were distinguishable from the adventitious (mouse) transcripts using a probe that detects their distinct 3'-UTR (GenBank accession number U82130) remained at a constant level (Fig. 3, middle). Given the observed dramatic decrease in endogenous TSG101 protein under experimental conditions (Fig. 2), in which endogenous mRNA production remained constant, the observed down-regulation of TSG101 protein production must necessarily be independent of any transcriptional control. To determine whether this posttranscriptional control occurs at the level of translation, we radioactively labeled total cellular protein with [35S]methionine and analyzed the proteins immunoprecipitated by excess anti-TSG101 antibody (Fig. 4A). In the absence of inducer, a single radioactive band whose rate of synthesis was unaltered by the induced synthesis of adventitious Flag-tagged TSG101 protein was observed, indicating that translation of the adventitious protein does not interfere with the synthesis of endogenous TSG101 and thus implying that the regulation of intracellular TSG101 levels within a narrow range is accomplished posttranslationally.

The notion that almost-constant steady-state levels of TSG101 are maintained within cells by a posttranslational mechanism was confirmed by pulse-chase experiments in which proteins synthesized in cells growing in the presence or absence of PA were labeled with [35S]methionine for 1 h, transferred to media containing excess unlabeled methionine, and then cultured for the times shown. As seen in Fig. 4B, after 8 h of nonradioactive chase of the methionine pulse, the combined intracellular level of labeled adventitious and endogenous TSG101 proteins was less than the level of endogenous TSG101 alone in noninduced cells, indicating that overexpression of adventitious TSG101 accelerates the turnover rate of the entire intracellular pool of TSG101 protein. These findings implicate enhanced protein decay as the mechanism that prevents intracellular accumulation of TSG101 during overexpression of the adventitious gene.

Fig. 5. Mutations at the COOH-terminal domain of the adventitious protein affect down-regulation of the endogenous TSG101 protein. A, summary of results for the tested DNA constructs, which are mutant derivatives of mouse TSG101 cDNA. These constructs were all introduced by transfection into Ecr293, and stable cell lines were established. UBC, TSG101 domain resembling ubiquitin-conjugating enzyme; PPPP, proline-rich domain; CC2, coiled coil domain, SB, the steadiness box defined in these experiments (see text); Δ, short in-frame deletions; F, Flag tag. The mutations are as follows: SD1-1 has a deletion of AAs 41–43; SD2-9 has a replacement of AAs 50–52 by Phe; SD3-17 has a deletion of AAs 193–195; SD4-28 has a replacement of AAs 273–276 by Asp; and SD5-59 has a replacement of AAs 348–351 by Gly. The deletion sites and fusion junctions involving the COOH-terminal regions are indicated in Fig. 7: LD1-18 has a deletion of 42 AAs at the COOH-terminal; Z-cPEP1 and Z-cPEP2 were generated by fusing E. coli LacZ with cPEP1 and cPEP2, respectively (see “Materials and Methods” for details). B, immunoblot analysis showing the effects of mutated adventitious TSG101 protein on the endogenous protein. Multiple stable transfectant cell lines were tested for each mutated construct, and all showed results similar to those seen here for one representative cell line. Cells were cultured with or without 5 μM PA induction for 24 h as shown. Total cellular protein was adjusted to the same concentration before assay of the induced and noninduced cell lines. Antibody against the full-length TSG101 protein was used in immunoblot analyses. C, induction time course for expression of LD1-18 in Ecr293. The protein amount was the same for each lane. The arrow represents endogenous TSG101 protein. D, induction time course for expression of fused proteins containing TSG101 COOH-terminal peptides. Antibody was specifically directed against the COOH-terminal peptide of TSG101 (2). Arrow, endogenous TSG101 protein; asterisk, internal control.
Fig. 6. Characterization of the SB. The last 95 AAs at the COOH-terminal end of human and mouse TSG101 proteins are aligned with homologous sequences from C. elegans and yeast. The PILEUP and PRETTY programs from the GCG package (25) were used for the alignment. Gap penalty is 10. Length penalty is 1. Plurality is 3. Uppercase letters represent the identical and conserved AA residues. CC2 and SB regions are indicated. Arrows define the cPEP1 and cPEP2 regions. cPEP1 was deleted in construct LD1-18. cPEP1 and cPEP2 were fused to LacZ in the constructs Z-cPEP1 and Z-cPEP2, respectively (Fig. 5). The dashed line shows the AAs deleted and replaced with glycine in construct SD5-59 (Figs. 5 and 6). The circled plus markers indicate the positively charged AA residues conserved in all four aligned sequences.

A Conserved Region within the COOH-terminal Domain Is Required for TSG101 Protein Autoregulation. To identify the TSG101 sequences involved in posttranslational regulation of the intracellular concentration, we constructed a set of in-frame short deletion mutations within murine TSG101 cDNA and isolated stably transfected Ecr293 cell clones containing these constructs (Fig. 5). Cell lines showing induced expression were obtained at a high frequency for all constructs except SD4-28, which produced detectable protein in only 5 of 40 clones. As seen in Fig. 5B, four of five TSG101 mutant proteins containing short in-frame deletions (i.e. constructs DS1-1, DS2-9, SD3-17, and SD4-28, respectively) retained the ability to down-regulate endogenous TSG101 protein after induction, whereas the product of construct SD5-59, in which a glycine residue replaced four AAs (AAs 348–351; Fig. 5), had little effect on the down-regulation of the endogenous protein. Confirmation that the COOH-terminal end of adventitious TSG101 is necessary for down-regulation of the endogenous protein was provided by our finding that the LD1-18 construct, which lacks 42 AAs at the COOH-terminal end of TSG101, totally lost this ability; additionally, unlike the full-length adventitious TSG101, it showed much less reduction of endogenous TSG101, suggesting that the mutant is defective in its inability to accelerate decay of the endogenous full-length protein.

DISCUSSION

Our results indicate that the steady-state level of TSG101 protein normally is maintained within a narrow range during cell growth by a mechanism that operates at the level of protein decay. Overexpression of adventitious Flag-tagged TSG101 protein resulted in down-regu-
lation of the endogenous protein without affecting the intracellular level of endogenous TSG101 mRNA, indicating that the observed autoregulation of TSG101 expression occurs posttranscriptionally. Pulse-chase experiments showed that overexpression of adventitious TSG101 leads to increased turnover of both the endogenous and adventitious TSG101 proteins, limiting the ability of TSG101 to accumulate within cells. Continued overproduction of adventitious TSG101, accompanied by continued accelerated turnover of the total intracellular TSG101 protein pool, results in effective replacement of the endogenous TSG101 protein by the adventitious protein.

These findings suggest a model in which the biological effects of TSG101 are modulated either by self-promoted proteolysis or participation with other cellular protein(s) in a proteolytic feedback control loop. The posttranslational autoregulation observed for TSG101 at the level of protein decay is reminiscent of the mechanism that prevents intracellular accumulation of the product of another tumor susceptibility gene, p53 (21). In that case, overproduction of p53 promotes the synthesis of the MDM2 protein, which in turn accelerates p53 degradation (22, 23). If TSG101 participates in an analogous feedback control loop with a second protein, its partner in the loop is also likely to be affected by alterations in TSG101 production.

The ability of overexpressed TSG101 to accelerate its own degradation is consistent with the proposal that TSG101 may have a role in proteolysis (14, 15). However, the COOH-terminal SB region that our studies show is necessary for normal regulation of the intracellular concentration of TSG101 is distinct from the ubiquitin-conjugase-like domain previously identified in the NH2-terminal region of TSG101. TSG101 derivatives lacking or mutated in the SB accumulate at several times the normal level while also not reducing the concentration of endogenous TSG101 protein, demonstrating a crucial role for this region in TSG101 autoregulation. However, truncated adventitious proteins containing only the SB fail to accomplish down-regulation of endogenous TSG101 (Fig. 6), indicating that the SB alone is insufficient. Interestingly, the SB and its flanking sequences are the most conserved regions of TSG101 homology among yeast, Caenorhabditis elegans, and mammals (Fig. 7), suggesting an evolutionarily preserved function in these disparate organisms. Sequence similarity in the SB is 69% between C. elegans and human or mouse and 56% between yeast and these mammals. Among the 45 AAs in the SB, there are 21 AAs (47%) conserved and 11 identical AAs (24%) in all four species.

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