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Proteasome-Mediated Degradation of the Vitamin D Receptor (VDR) and a Putative Role for SUG1 Interaction With the AF-2 Domain of VDR

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Abstract The AF-2 helix of nuclear receptors is essential for ligand-activated transcription, and it may function to couple the receptor to transcriptional coactivator proteins. This domain also contacts components of the proteasome machinery, suggesting that nuclear receptors may be targets for proteasome-mediated proteolysis. In the present study, we demonstrate that mSUG1 (P45), a component of the 26S proteasome, interacts in a 1,25-$(OH)_2D_3$–dependent manner with the AF-2 domain of the vitamin D receptor (VDR). Furthermore, treatment of ROS 17/2.8 osteosarcoma cells with the proteasome inhibitors MG132 or β-lactone increased steady-state levels of the VDR protein. In the presence cycloheximide (10 μg/ml), the liganded VDR protein was degraded with a half-life of approximately 8 h, and this rate of degradation was completely blocked by 0.05 mM MG132. The role of SUG1-VDR interaction in this process was investigated in transient expression studies. Overexpression of wild-type mSUG1 in ROS17/2.8 cells generated a novel proteolytic VDR fragment of approximately 50 kDa, and its production was blocked by proteasome inhibitors or by a nonhydrolyzable ATP analog. Parallel studies with SUG1(K196H), a mutant that does not interact with the VDR, did not produce the 50 kDa VDR fragment. Functionally, expression of SUG1 in a VDR-responsive reporter gene assay resulted in a profound inhibition of 1,25-$(OH)_2D_3$–activated transcription, while expression of SUG1(K196H) had no significant effect in this system. These data show that the AF-2 domain of VDR interacts with SUG1 in a 1,25-$(OH)_2D_3$–dependent fashion and that this interaction may target VDR to proteasome-mediated degradation as a means to down-regulate the 1,25-$(OH)_2D_3$–activated transcriptional response. J. Cell. Biochem. 71:429–440, 1998.

Key words: proteasome; VDR; SUG1; AF-2 domain; 1,25-$(OH)_2D_3$

The genomic effects of 1,α25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$) are mediated by the vitamin D receptor (VDR), which is a member of nuclear receptor superfamily and functions as a ligand-induced transcription factor [Evans, 1988; Tsai and O'Malley, 1994]. VDR forms heterodimers with retinoid X receptor (RXR), binds to vitamin D responsive elements (VDRE) in the promoters of vitamin D–responsive genes, and alters the rate of transcription of selected genes [Darwish and DeLuca, 1993; MacDonald et al., 1994; Whitfield et al., 1995]. The 1,25-$(OH)_2D_3$ ligand promotes heterodimerization with RXR and binding to VDRE [Freedman, 1992; MacDonald et al., 1994], and it may also induce a conformational change that alters the VDR AF-2 domain for interaction with various nuclear receptor coactivator proteins [Masuyama et al., 1997]. Several putative cofactor proteins, including steroid hormone receptor coactivator-1 (SRC-1) [Onate et al., 1995], receptor interacting protein 140 (RIP140) [Cavailles et al., 1995], glucocorticoid receptor interacting protein 1 (GRIP1) [Hong et al., 1997], and suppresser for gal 1 (SUG1) [vom Bauer et al., 1996], have been shown to interact with VDR in

Abbreviations used: ATPγS, adenosine 5′-O-(3-thiotriphosphate); DMSO, dimethyl sulfoxide; E64, trans-epoxysuccinyl-L-leucylamido(4-guanidino)-butane; GRIP1, glucocorticoid receptor interacting protein 1; LLM, N-acetyl-leu-leu-methioninal; LLnL, N-acetyl-leu-leu-norleucinal; MG132, Z-leu-leu-leu-H; RIP140, receptor interacting protein 140; RXR, retinoid X receptor; SRC-1, steroid hormone receptor; SUG1, suppressor for gal; VDR, vitamin D receptor; VDRE, vitamin D responsive element; β-lactone, clasto-lactacystin β-lactone; 1,25-$(OH)_2D_3$, 1,α25-dihydroxyvitamin D_3.

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a ligand- and AF-2–dependent manner [Hong et al., 1997; Masuyama et al., 1997]. Although a functional role for these putative coactivators in VDR-mediated transcription is not well established, their expression augments ligand-activated transcription by a variety of nuclear receptors, clearly suggesting a transcriptional coactivator role [Cavailles et al., 1995; Hong et al., 1997; Onate et al., 1995]. In contrast, a similar role for SUG1 in this complex mechanism has not been forthcoming. Although yeast SUG1 was originally identified as a transcription factor [Swaffield et al., 1992], recent evidence indicates that this protein is a component of the 26S proteasome complex [Rubin et al., 1996].

The proteasome is a major cytosolic and nuclear protease complex that is responsible for an ATP-dependent, extralysosomal proteolytic pathway. This complex is responsible for the degradation of most cellular proteins, and the proteasome activity is necessary for cell viability [Coux et al., 1996; Rock et al., 1994; Tanaka, 1995]. The proteasome is highly conserved throughout eukaryotic evolution, and it exists as two major complexes: the 20S proteasome, which contains multiple peptidase activities, and the 26S proteasome, which contains the 20S subunit as well as a 19S regulatory complex composed of multiple ATPases and components necessary for binding protein substrates [Coux et al., 1996; Tanaka, 1995]. Ubiquitinylation of the substrate protein is an important step in proteasome-mediated degradation of most proteins. However, degradation of several proteins, such as casein and ornithine decarboxylase, do not required ubiquitinylation [Coux et al., 1996; Hershko and Ciechanover, 1992]. To date, a wide variety of substrates for the proteasome has been described [reviewed in Coux et al., 1996; Hilt and Wolf, 1995], including rate-limiting enzymes such as ornithine decarboxylase [Murakami et al., 1992], transcriptional regulators such as c-Jun [Trier et al., 1994], p53 [Scheffner et al., 1990], and NF-kB [Palombella et al., 1994], critical regulatory proteins such as cyclins [Ghislain et al., 1993], and tyrosine kinase receptors [Jeffers et al., 1997]. The involvement of the proteasome in nuclear receptor turnover is not presently known.

Using the yeast two-hybrid system, we and others have isolated components of the proteasome pathway as proteins that interact selectively with VDR and other nuclear receptors [Lee et al., 1995; Masuyama et al., 1997; vom Bauer et al., 1996]. One clone that is consistently isolated in our screens is SUG1 (also termed TRIP1 for thyroid receptor interacting protein) [Lee et al., 1995]. SUG1 interacts with the AF-2 domain of various nuclear receptors in a ligand-dependent fashion [Masuyama et al., 1997; vom Bauer et al., 1996]. SUG1 is completely identical to p45, a ATPase subunit of the proteasome [Akiyama et al., 1995]. The fact that SUG1 selectively interacts with nuclear receptors led us to investigate whether the VDR was a target for proteasome degradation. In this study, we demonstrate that VDR is degraded by the proteasome and that overexpression of SUG1 selectively altered VDR proteolysis. These data suggest a general mechanism for receptor downregulation that may involve proteasome-mediated proteolysis via SUG1 interaction with the AF-2 domain of the nuclear receptors.

MATERIALS AND METHODS

Preparation of Two-Hybrid Expression Vectors and cDNA Library Screening

All two-hybrid plasmids constructs used the pAS1 [Hannon et al., 1993] and pAD-GAL4 yeast expression vectors (Stratagene, La Jolla, CA). The AS1-VDR construct and various AS1-VDR mutant constructs were previously described [MacDonald et al., 1995; Masuyama et al., 1997]. A point mutation, K196H, was introduced into the mouse SUG1 cDNA with oligonucleotide-directed mutagenesis [Carter, 1987; Deng and Nickoloff, 1992]. The K196H mutation was confirmed by DNA sequencing and subcloned into the pAD-GAL4 vector to examine in the two-hybrid assay. The MC3T3-E1 cell cDNA library in the pAD-GAL4 vector was a kind gift from Dr. R. St-Arnaud (Montreal, Canada). For cDNA library screening, the library was cotransformed with pAS1-VDR (93–427) into the yeast strain Hf7c, which was made competent with lithium acetate [Gietz et al., 1991]. Transformants were plated on media lacking leucine, tryptophan, and histidine (SC-leu-trp-his) and containing 10^{-8} M 1,25-$(OH)_2D_3$ and 10 mM 3-amino-1,2,4-triazole. Histidine positive colonies were assayed for β-galactosidase expression using a colony lift filter assay [Hannon et al., 1993].

β-Galactosidase Assays

The wild-type and mutant pAD-mSUG1 were cotransformed with wild-type and mutant pAS1-VDR into the yeast strain Hf7c. Transformants were plated on media lacking leucine and tryptophan (SC-leu-trp) and were grown for 4 days at 30°C to select for yeast that had acquired both plasmids. Triplicate independent colonies from each plate were grown overnight in 2 ml of SC-leu-trp with or without the indicated concentrations of 1,25-$(OH)_2D_3$. Cells were harvested and assayed for β-galactosidase activity as described [Fagan et al., 1994].

Inhibitors

Z-leu-leu-leu-H (MG132) was purchased from Peptides International Inc. (Louisville, KY). N-acetyl-leu-leu-norleucinal (LLnL), N-acetyl-leu-leu-methioninal (LLM), trans-epoxysuccinyl-L-leucylamido(4-guanidino)-butane (E64), adenosine 5′-O-(3-thiotriphosphate) (ATPγS), and dimethyl sulfoxide (DMSO) were purchased from Sigma Chemical Co. (St. Louis, MO). Clasto-lactacystin β-lactone (β-lactone) was obtained from Calbiochem-Novabiochem Co. (San Diego, CA). All inhibitors were maintained in DMSO at a final concentration of 50 mM and stored at -20°C. All inhibitors and 1,25-$(OH)_2D_3$ ligand were added simultaneously to the culture media at the indicated concentrations.

Nuclear Extraction and Western Analysis

Nuclear extracts were obtained from ROS 17/2.8 cells essentially by the method of Shapiro et al. [1988]. The subconfluent cells were washed with ice-cold phosphate buffered saline (PBS) twice and harvested with a cell scraper. The cell pellets were resuspended in 2 volumes of hypotonic buffer (10 mM Hepes-KOH, pH 7.9, 0.75 mM spermidine, 0.15 mM spermine, 0.1 mM EGTA, 0.1 mM EDTA, 10 mM KCl, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 15 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin A), incubated on ice for 10 min, and disrupted with a Dounce homogenizer. The homogenate was adjust to 1.3 M sucrose and centrifuged at 16,000*g* for 30 sec. The crude nuclear pellet was resuspended in 2 volumes of nuclear resuspension buffer (20 mM Hepes, pH 7.9, 0.75 mM spermidine, 0.15 mM spermine, 0.2 mM EGTA, 0.2 mM EDTA, 25% glycerol, 2 mM dithiothreitol), and saturated ammonium sulfate was added to 0.1 volume. The crude extract was incubated with rocking for 30 min and centrifuged at 100,000*g* for 90 min at 4°C. Solid ammonium sulfate was added to a concentration of 0.33g/ml, incubated with rocking for 20 min, and centrifuged at 100,000*g* for 20 min. The pellet was resuspended in 0.3 ml of dialysis buffer (20 mM Hepes pH 7.9, 0.2 mM EGTA, 0.2 mM EDTA, 20% glycerol, 100 mM KCl, 2 mM DTT), dialyzed against 2 × 300 ml of dialysis buffer, and stored at -80°C. Equivalent amounts of nuclear protein from each extract were solubilized in SDS buffer and analyzed by Western blot analysis as previously described [MacDonald et al., 1995] using rat monoclonal antibody 9A7γ for VDR, rabbit polyclonal antibody for transcription factor IIB (TFIIB) (Santa Cruz Biotechnology Inc., Santa Cruz, CA), and rabbit antiserum for SUG1 (a kind gift from Dr. D. Nathans, Johns Hopkins University, Baltimore, MD). Western blots were quantitated using a Scanning Imager 300SX instrument (Molecular Dynamics, Sunnyvale CA) and ImageQuant (version 3.3) software.

Transient Transfection Studies

The VDRE^4-TATA-GH, VDRE^4-TK-GH, BGP (-1000)-GH, and RSV-GH were described previously [MacDonald et al., 1993; Masuyama et al., 1997; Terpening et al., 1991]. Wild-type and mutant (K196H) mSUG1 constructs were subcloned into the pcDNA3 expression vector (Invitrogen Co., San Diego, CA). The pSG5-VDR expression plasmid was described previously [Hsieh et al., 1991; MacDonald et al., 1993]. COS-7 cells were cotransfected with reporter gene constructs (VDRE^4-TATA-GH, VDRE^4-TK-GH), receptor expression vectors (pSG5-VDR), and mSUG1 expression vectors (WT, K196H, or pcDNA expression vector alone). ROS 17/2.8 cells, which express native VDR at approximately 20,000 copies per cell, were cotransfected with reporter gene constructs (VDRE^4-TATA-GH, VDRE^4-TK-GH, or BGP(-1000)-GH) and mSUG1 expression vectors. For introducing plasmids into COS-7 cells, the amount of total DNA was kept constant at 10 μg by adding pTZ18U (U.S. Biochemical, Cleveland, OH) as a carrier plasmid, and the cells were transfected by standard calcium phosphate coprecipitation procedures as described previously [MacDonald et al., 1993]. In all transfections using ROS17/2.8 cells, liposome-mediated transfections were accomplished with lipofectamine

(Life Technologies Inc., Gaithersburg, MD) according to the manufacturer's protocol. Transfected cells were treated with 10^{-8} M 1,25-$(OH)_2D_3$ or ethanol vehicle for the indicated period, and the amount of secreted GH was determined with a radioimmunoassay kit (Nichols Institute, San Juan Capistrano, CA).

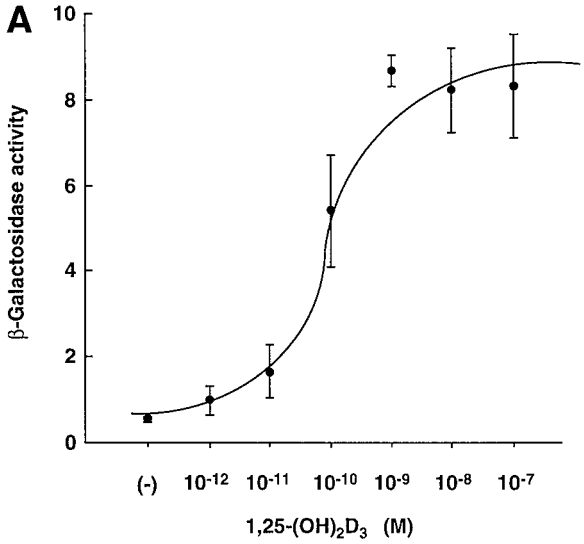
RESULTS

Isolation of mSUG1 and Ligand- and AF-2–dependent Interaction Between VDR and mSUG1

Two-hybrid strategies were used in this study to identify proteins that interact with the ligand binding domain (LBD) of VDR in a ligand-dependent manner. The bait construct in this screen was AS1-VDR(93–427), which contained the Gal4 DNA binding domain (amino acids 1–147) fused to the LBD of VDR (amino acids 93–427) [MacDonald et al., 1995]. A mouse osteoblastic MC3T3 cell cDNA library in the yeast multicopy expression vector pAD-GAL4 was examined in this screen [Masuyama et al., 1997]. Interaction between AS1-VDR and fusion proteins generated from the cDNA library was monitored on selective media lacking histidine and containing 10^{-8} M 1,25-$(OH)_2D_3$ as described previously [Masuyama et al., 1997]. Several cDNA clones that interacted with VDR in the presence of 1,25-$(OH)_2D_3$ were obtained [Masuyama et al., 1997]. DNA sequence analysis identified several of these clones as full-length mSUG1 [vom Bauer et al., 1996]. As illustrated in Figure 1A, mSUG1 interacted with VDR in a concentration-dependent manner with increasing amounts of 1,25-$(OH)_2D_3$ in this two-hybrid system. Half maximal β-galactosidase activity occurred at approximately 10^{-10} M 1,25-$(OH)_2D_3$, thus showing strong correlation with the apparent Kd for the VDR-1,25-$(OH)_2D_3$ complex. Deletion analysis of VDR revealed that gross truncations that abolished ligand binding [pAS1-VDR(281–427) or pAS1-VDR(93–386)] also disrupted ligand-dependent interaction of VDR with SUG1 (Fig. 1B). However, deletion of the AF-2 motif in the pAS1-VDR(93–403) construct retains significant ligand binding [Masuyama et al., 1997; Nakajima et al., 1994] but was incapable of SUG1 interaction. Moreover, point mutations in the AF-2 motif also eliminated ligand-dependent interaction of VDR with SUG1 [Masuyama et al., 1997], and the minimal AF-2 domain of VDR alone [pAS1-VDR(408–427)] was sufficient for the interaction with SUG1 (Fig. 1B). These data suggest that the interaction between VDR and SUG1 is dependent on ligand and is mediated in part through the AF-2 domain of VDR.

Effect of Proteasome Inhibitors on VDR Protein Levels in ROS 17/2.8 Cells

Because mSUG1 is completely identical to p45, a component of the regulatory subunit of the proteasome [vom Bauer et al., 1996], we reasoned that VDR may be a target for proteasome-mediated degradation. Thus, VDR protein levels were qualitatively examined in ROS 17/2.8 cells that were exposed to a variety of proteasome inhibitors. Interestingly, VDR protein levels were increased eightfold in the presence of 0.05 mM MG132, which strongly inhibits proteasome activities [Rock et al., 1994] (Fig. 2A, lane 2). In contrast, steady-state levels of transcription factor IIB (TFIIB) were unaffected by MG132 treatment under these conditions. Similar results were observed when whole cell extracts were examined following a direct solubilization of the cell monolayers in SDS-PAGE sample buffer (data not shown). Several other inhibitors of proteasome or lysosomal proteases were examined to confirm whether the increase of VDR protein is due to an inhibition of the proteasome pathway. Treatment with LLM or LLnL, which are also proteasome-selective [Rock et al., 1994], increased VDR protein levels approximately four- or sixfold, respectively (Fig. 2A, lanes 3,4). In contrast, VDR levels did not change in the response to the lysosomal protease inhibitor (E64) (Fig. 2A, lane 5) [Rock et al., 1994]. The inhibitors did not significantly affect TFIIB protein levels under these conditions. We also tested β-lactone, which is a product of lactacystin hydrolysis and is the most selective inhibitor of proteasome degradation reported thus far [Dick et al., 1996]. Treatment of ROS17/2.8 cells with β-lactone resulted in VDR protein accumulation in both the presence and absence of ligand (Fig. 2B, lanes 2,4); however, the effect was more pronounced with liganded VDR (a fourfold vs. a sixfold increase, respectively). VDR degradation was also examined in the absence of ongoing protein synthesis (Fig. 2C). ROS17/2.8 cells were treated with cycloheximide (10 μg/ml), and the effect of MG132 on VDR degradation was determined. In the absence of the 1,25-



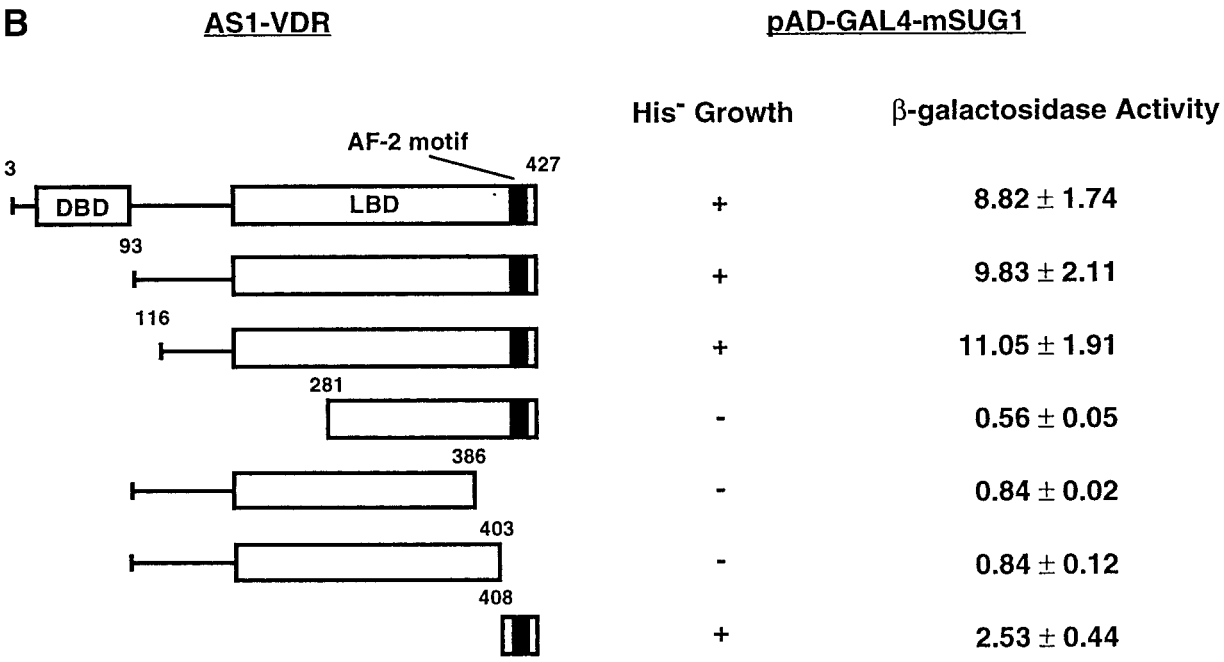


Fig. 1. Ligand-dependent interaction between VDR and mSUG1 in a two-hybrid system. **A:** 1,25-$(OH)_2D_3$– dependent interaction between VDR and mSUG1. Yeast expressing the pAS1-VDR(93–427) and pAD-mSUG1 two-hybrid plasmids were grown for 24 h at 30°C in the absence and presence of increasing concentrations of 1,25-$(OH)_2D_3$. VDR-mSUG1 interaction was assessed in a β-galactosidase assay. Results are presented as the mean ± SD of triplicate independent cultures. **B:** The AF-2 domain of VDR is required for interaction with mSUG1. The pAD-mSUG1 plasmid was cotransformed with various pAS1-VDR derivatives into the yeast strain Hf7c. Relative growth of yeast on histidine-deficient plates was assessed after 4 days at 30°C. mSUG1 interaction with each VDR derivative was quantitated in a β-galactosidase assay after overnight growth in a selection media (SC-leu-trp) in presence of 10^{-8} M 1,25-$(OH)_2D_3$. Results are presented as the mean ± SD of triplicate independent cultures.

$(OH)_2D_3$ ligand, the VDR protein was rapidly degraded ($t_{1/2} < 4$h), and the MG132 proteasome inhibitor completely blocked this degradation (Fig. 2C, lanes 1–6). Degradation of liganded VDR proceeded at a somewhat slower rate ($t_{1/2} > 8$ h), and this is consistent with previous findings showing a modest ligand-induced stability of the receptor [Arbour et al., 1993; van den Bemd et al., 1996; Wiese et al., 1992]. Again, this degradation in the absence of protein synthesis was completely blocked by the MG132 inhibitor (Fig. 2C, lanes 7–14).

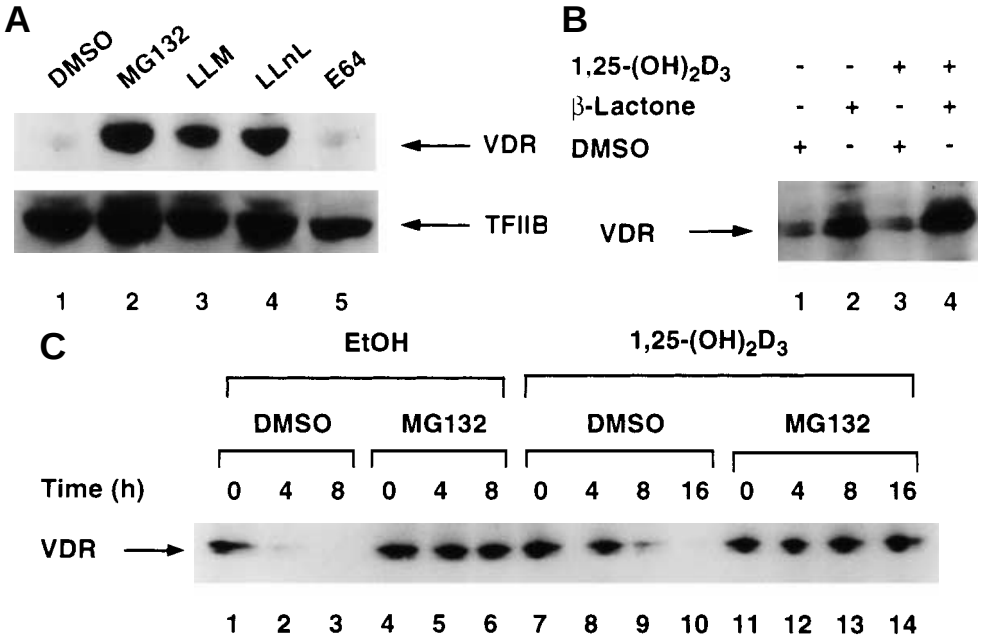


Fig. 2. The effect of proteasome inhibitors on VDR protein levels in ROS 17/2.8 cells. **A:** Subconfluent ROS 17/2.8 cells were treated with dimethylsulfoxide (DMSO) or with 0.05 mM of various inhibitors for 3 h. E64, trans-epoxysuccinyl-L-leucylamido(4-guanidino)-butane); LLM, N-acetyl-leu-leu-methioninal; LLnL, N-acetyl-leu-leu-norleucinal; MG132, Z-leu-leu-leu-H. Nuclear extracts were prepared as described in Methods. Equivalent amounts of each extract were resolved by 10% SDS-PAGE, and relative VDR and TFIIB protein levels were determined by Western blotting using anti-VDR antibody (9A7γ) or anti-TFIIB antibody. **B:** Subconfluent ROS 17/2.8 cells were treated with dimethylsulfoxide (DMSO) or with 0.02 mM of β-lactone for 3 h in the presence or absence of 10^{-8} M 1,25-$(OH)_2D_3$, and nuclear extracts were prepared. VDR protein levels were determined as described above. **C:** Subconfluent ROS 17/2.8 cells were treated with cycloheximide (10 μg/ml media) for 10 min prior to inhibitor and ligand addition. Then cells were treated with DMSO or with 0.05 mM of MG132 for the indicated period in the presence or absence of 10^{-8} M 1,25-$(OH)_2D_3$. Nuclear extracts were prepared and VDR protein levels examined as described. This level of cycloheximide inhibited >95% of ^{35}S-labeled methionine incorporation into trichloroacetic acid (TCA)–precipitated protein (data not shown).

SUG1 Overexpression Enhances VDR Proteolysis in ROS 17/2.8 Cells

To test whether the SUG1-VDR interaction is involved in VDR degradation in this system, wild-type SUG1 and SUG1 (K196H), a mutant that does not interact with VDR [vom Bauer et al., 1996], were transiently overexpressed in ROS 17/2.8 cells, and their effect on VDR protein levels was examined in Western immunoblots (Fig. 3A). In the absence of ligand, overexpression of wild-type and mutant SUG1 did not significantly affect VDR protein levels (Fig. 3A, lanes 1–3). However, in the presence of 10^{-8} M 1,25-$(OH)_2D_3$, a novel proteolytic fragment of the VDR (asterisk in Fig. 3A, lane 5) was observed when ROS 17/2.8 cells were transfected with the wild-type SUG1 expression vector. The novel VDR fragment was less apparent in cells transfected with the SUG1 (K196H) mutant. These data suggest that a ligand-dependent interaction between VDR and SUG1 resulted in the generation of this proteolytic fragment of VDR. Related studies using the MG132 inhibitor indicated that the VDR proteolytic fragment was generated by proteasome-mediated degradation. As shown in Figure 3B (lane 3), 0.05 mM MG132 completely blocked the formation of this 1,25-$(OH)_2D_3$– and SUG1-dependent fragment. The production of this proteolytic fragment could also be blocked by treating the cells with the nonhydrolyzable ATP analog, ATPγS (Fig. 3B, lane 5), indicating that both proteasome activity and ATP hydrolysis were required for the generation of this SUG1-dependent proteolytic fragment of the VDR in this system.

Suppression of VDR-Mediated Transcription by Overexpressed Wild-Type mSUG1

To examine the effect of mSUG1 on 1,25-$(OH)_2D_3$/VDR–mediated transactivation, mSUG1 was expressed in a vitamin D–responsive tran-

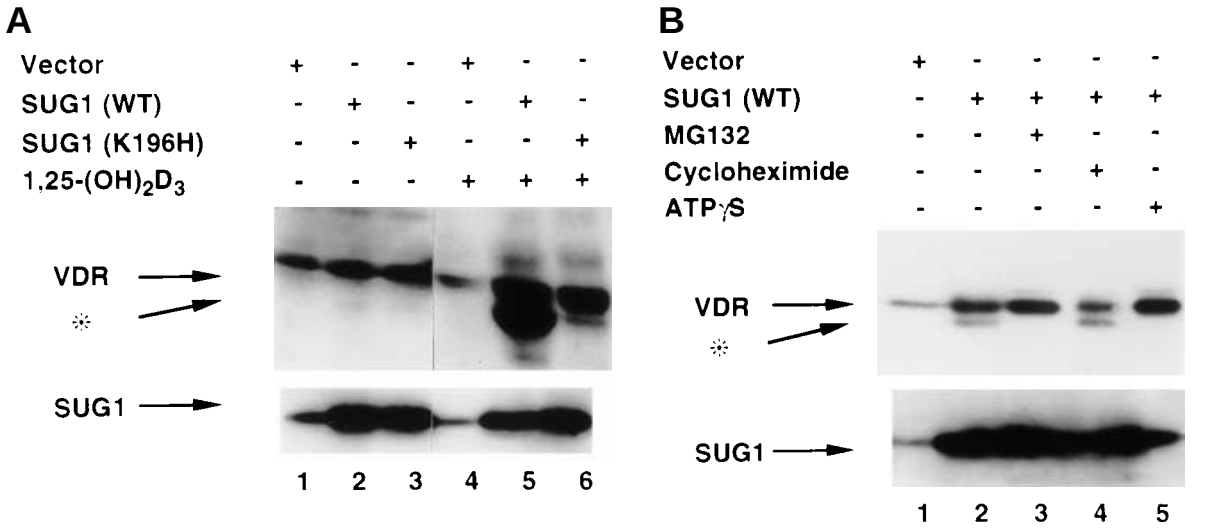


Fig. 3. The enhancement of VDR proteolysis by SUG1 overexpression. **A:** ROS17/2.8 cells on 150 mm plates were transfected with 10 µg of pcDNA3 parent expression plasmid (**lanes 1 and 4**) or pcDNA3 derivatives that express wild-type or mutant mSUG1 (**lanes 2 and 5** or **lanes 3 and 6,** respectively). The cells were treated with ethanol vehicle or 10^{-8} M 1,25-$(OH)_2D_3$ for 6 h, and nuclear extracts were prepared. VDR and SUG1 protein levels were examined as described in Fig. 2. **B:** ROS17/2.8 cells were transfected with 10 µg of pcDNA3 parent expression plasmid (lane 1) or pcDNA3 derivatives that express wild-type mSUG1 (lanes 2–5). The cells were treated with 10^{-8} M 1,25-$(OH)_2D_3$ and exposed to 0.05 mM of MG132, 10 µg/ml cycloheximide, or 5 mM ATPγS for 6 h, and nuclear extracts were prepared. VDR and SUG1 protein levels were examined as described in Fig. 2. The proteolytic VDR fragment is indicated by an asterisk in A and B.

sient gene expression system. A VDR expression plasmid (SG5-VDR) and vitamin D responsive reporter gene constructs (VDRE4-TATA-GH and VDRE4-TK-GH) were introduced into COS-7 cells in the absence or presence of an expression vector that generates mSUG1 (pcDNA3-mSUG1) (Fig. 4A). Expression of mSUG1 suppressed vitamin D–mediated transactivation of both reporter constructs, but it had no effect on basal transcription. This same inhibitory effect of mSUG1 on vitamin D–mediated transcription was also observed in an authentic vitamin D responsive osteoblast-like target cell line, ROS 17/2.8. Vitamin D–mediated expression of both the artificial VDRE4-TATA-GH reporter and the reporter construct driven by the native osteocalcin promoter (BGP(-1000)-GH) were inhibited by SUG1 expression in ROS 17/2.8 cells. This effect depended on the amount of mSUG1, and the maximal suppression was about 70% compared to similar transfections with the parent expression vector (pcDNA3) (data not shown). SUG1 expression did not affect general transcriptional processes when RSV-GH and SV40-GH reporter constructs were examined in ROS 17/2.8 cells (Fig. 4A). The suppressive effect on 1,25-$(OH)_2D_3$–dependent transcription was due to an interaction between VDR and SUG1 because expressing SUG1 (K196H), which does not interact with VDR [vom Bauer et al., 1996], had no significant effect on vitamin D–mediated transactivation in this system (Fig. 4B).

DISCUSSION

Sug1 was identified originally as an essential factor in yeast that had properties consistent with those of a transcriptional mediator protein [Swaffield et al., 1992]. For example, SUG1 mutants suppress the effects of GAL4 activation domain mutants in yeast [Swaffield et al., 1992], and SUG1 interacts with a number of transcription factors, including the GAL4 and VP16 activation domains [Swaffield et al., 1995], TATA binding protein [Swaffield et al., 1995; vom Bauer et al., 1996], and TFIIH [Weeda et al., 1997]. More recently, SUG1 was shown to express DNA helicase activity [Fraser et al., 1997]. The murine homolog of yeast SUG1 was isolated in a two-hybrid screen as a protein that interacts in a ligand-dependent fashion with the thyroid hormone receptor [Lee et al., 1995] as well as several other nuclear receptors [vom Bauer et al., 1996]. Cumulatively, these data suggest that the putative SUG1 transcriptional mediator might couple liganded nuclear receptors to the transcriptional machinery and thus facilitate hormone-activated transcription. However, a functional role for SUG1 as a mediator or coactivator protein in nuclear receptor-

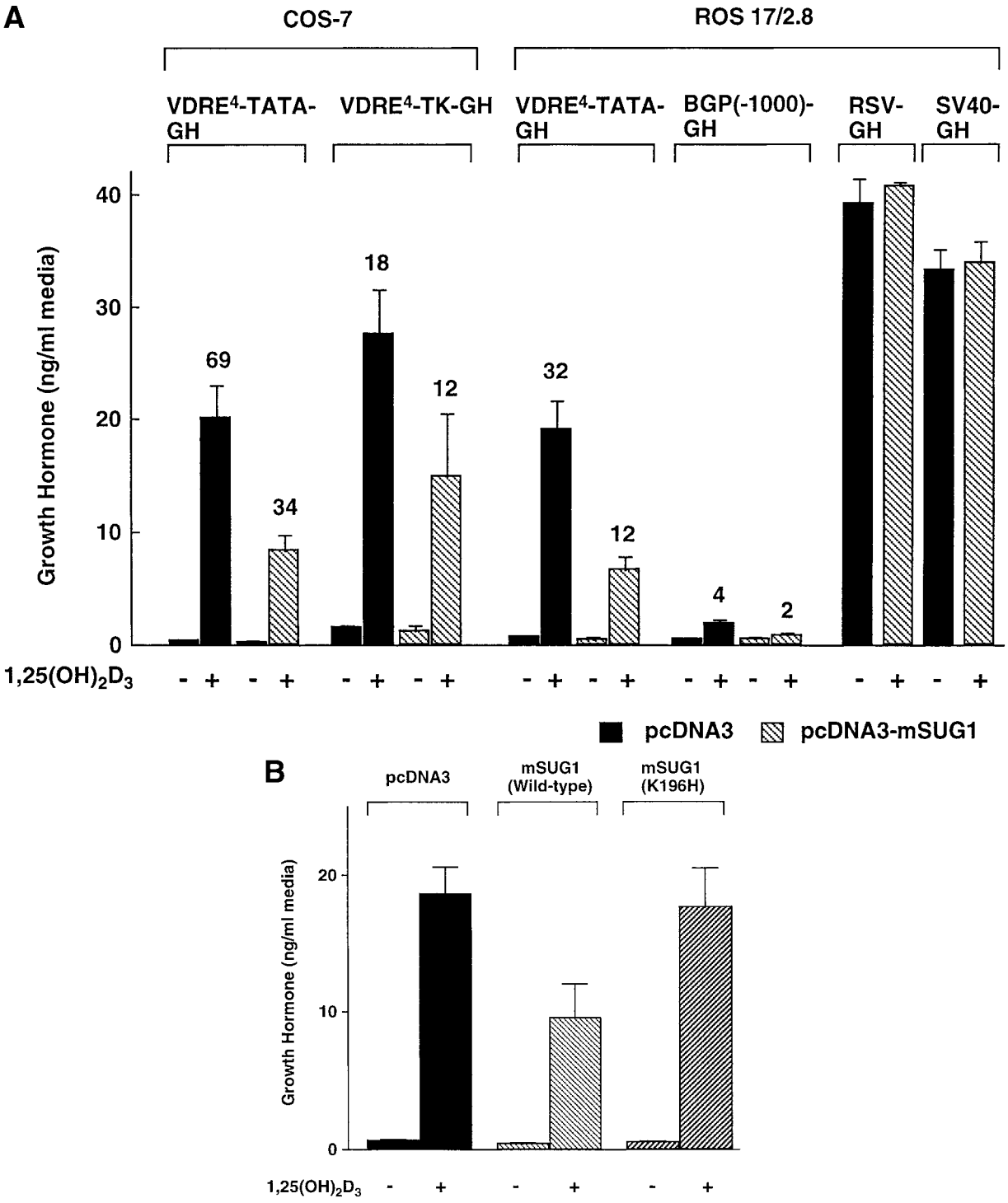


Fig. 4. Suppression of 1,25-$(OH)_2D_3$/VDR-mediated transactivation by wild-type mSUG1. **A:** COS-7 cells were transfected with 2 μg of the $VDRE^4$-TATA-GH or the $VDRE^4$-TK-GH reporter gene construct together with 50 ng of the VDR expression plasmid. The cells were also transfected with 1 μg RSV-GH or 2 μg SV40-GH. ROS17/2.8 cells were also cotransfected with 2 μg of $VDRE^4$-TATA-GH or BGP(-1000)-GH reporter gene constructs. These groups also received 1 μg of pcDNA3 parent expression plasmid or pcDNA3 derivatives that express wild type mSUG1. The cells were treated with ethanol vehicle (−) or with 10^{-8} M 1,25-$(OH)_2D_3$ (+) for 24 h. Growth hormone secreted into the media was quantitated using an immunoassay kit. The results represent the mean ± SD of triplicate determinations, and the number above each bar represents fold activation relative to the ethanol-treated control group. **B:** COS-7 cells were cotransfected with 2 μg of $VDRE^4$-TATA-GH reporter gene constructs, 1 μg of the mSUG1 expression plasmids (wild-type or K196H), or empty vector and 50 ng of pSG5–wild-type VDR. The cells were treated and growth hormone secretion quantitated as described for panel A.

dependent transcription has not been forthcoming. In contrast to a transcriptional mediator role, biochemical fractionation studies show that ySUG1 is tightly coupled to the 26S proteasome complex [Rubin et al., 1996]. Moreover, cloning of the murine homologue of SUG1 (mSUG1) [vom Bauer et al., 1996] revealed that it is completely identical to p45, an ATPase subunit of the 26S proteasome complex [Akiyama et al., 1995]. Convincing evidence for a role of SUG1 in proteasome action was indicated in yeast with a mutant SUG1 allele which accumulates ubiquitinylated proteins that are normally degraded by the proteasome [Ghislain et al., 1993]. Thus, the interaction of SUG1 with nuclear receptors and other transactivator proteins may be related to receptor turnover or degradation rather than functioning in the transactivation process per se.

Our current data support this concept. Using a VDR bait construct in a yeast two-hybrid screen of a MC3T3 osteoblast library, we isolated the full-length cDNA encoding mSUG1 as a protein that interacts with VDR in a 1,25-$(OH)_2D_3$–dependent manner [Masuyama et al., 1997]. Although several other putative coactivators, including SRC-1 and RIP140, were isolated in this same screen [Masuyama et al., 1997], mSUG1 did not display coactivator activity in a 1,25-$(OH)_2D_3$–mediated transient gene expression system. Indeed, transient overexpression of wild-type SUG1 negatively impacted VDR-mediated transcription in this system (Fig. 4A). Presumably interactions between SUG1 and the AF-2 domain of VDR were required for this downregulation because SUG1 (K196H), a mutant that does not interact with the VDR or with other nuclear receptors [vom Bauer et al., 1996], had no affect on 1,25-$(OH)_2D_3$ responsiveness in this system. One possibility is that a simple competition may occur between overexpressed SUG1 and other native AF-2 interacting proteins (e.g., coactivators) that limits their interaction with VDR, thus compromising the transcriptional response. Alternatively, a more active role for the VDR-SUG1 interaction may be to direct the VDR, and perhaps other nuclear receptors, toward a proteolytic pathway through the nuclear proteasome and thereby downregulate hormone receptor activity via receptor degradation.

Several lines of evidence support a role for the VDR-SUG1 interaction in receptor turnover by the proteasome. First, a variety of relatively selective inhibitors of the proteasome pathway, including MG132 and β-lactone, dramatically increased the steady-state levels of native VDR protein in nuclear extracts obtained from ROS 17/2.8 osteosarcoma cells. This effect was due at least in part to decreased degradation of the VDR as indicated by the cycloheximide studies in which MG132 treatment completely blocked the degradation of native VDR in ROS 17/2.8 nuclei (Fig. 2C). Secondly, overexpression of wild-type SUG1 in ROS17/2.8 cells resulted in the appearance of a truncated VDR derivative of approximately 50 kDa compared to the 52 kDa intact VDR (Fig. 3A). Here the VDR-SUG1 interaction was also required for the formation of this proteolytic derivative because overexpressing the SUG1 (K196H) mutant did not produce a similar effect. Moreover, the interaction between VDR and SUG1 requires the presence of the 1,25-$(OH)_2D_3$ ligand since we did not observe the 50 kDa proteolyzed VDR in SUG1-transfected ROS 17/2.8 cells in the absence of ligand. Finally, the MG132 proteasome inhibitor abolished the formation of the truncated VDR indicating that this 1,25-$(OH)_2D_3$– and SUG1-dependent product was the result of proteasome-mediated proteolysis. Cumulatively, these data suggest that SUG1 interacts with the AF-2 domain of liganded VDR and targets, either directly or indirectly, the VDR to degradation by the proteasome machinery.

An intriguing aspect of this potential mechanism is that two functionally discrete classes of proteins involved in two antagonistic roles in nuclear receptor action (i.e., transactivation vs. receptor degradation) may compete for similar binding sites on the receptor. The AF-2 domain of the nuclear receptors was defined on the basis of its central importance in ligand-activated transcription. The ligand-dependent activity is presumably mediated by coactivator proteins such as SRC-1, RIP140, and GRIP1 which interact in a ligand-dependent fashion with the AF-2 domain and facilitate nuclear receptor-dependent transcription by mechanisms that are poorly understood at present [Cavailles et al., 1995; Hong et al., 1997; Horwitz et al., 1996; Onate et al., 1995]. That the AF-2 domain is also the target for interaction with factors that eventually result in the degradation or proteolysis of the active nuclear form suggests a potential regulatory role of the proteasome in ligand-activated transcription. Al-

though it is well appreciated that downregulation of vitamin D–dependent transcription occurs through hydroxylation of the bioactive 1,25-$(OH)_2D_3$ metabolite by the 24-hydroxylase, degradation of the liganded VDR complex through proteolysis has the potential to rapidly and directly inactivate the hormone-dependent transcriptional response. It is possible that a single proteolytic cleavage of the VDR, as in the elimination of the 2 kDa carboxyl terminus containing the AF-2 domain, would be sufficient to completely inactivate the transcriptional response. We and others have shown that deletion of this domain from VDR and other nuclear receptors inactivates receptor-mediated transcription [Barettino et al., 1994; Danielian et al., 1992; Durand et al., 1994; Masuyama et al., 1997; Nakajima et al., 1994]. Therefore, it will be important in subsequent studies to determine the precise nature of the SUG1- and 1,25-$(OH)_2D_3$–dependent proteolytic derivative of VDR observed in the osteosarcoma cells.

The 1,25-$(OH)_2D_3$ ligand modestly increases the stability of the VDR, as demonstrated in several cell systems [Arbour et al., 1993; van den Bemd et al., 1996; Wiese et al., 1992], and this observation is reproduced here in the protein degradation analysis presented in Figure 2C. The 1,25-$(OH)_2D_3$ ligand induces a conformational change in VDR that makes the receptor more resistant to a variety of proteases in in vitro systems [Liu et al., 1997; Peleg et al., 1995]. Moreover, the crystal structures of related receptors (liganded retinoic acid receptor and thyroid hormone receptor vs. unliganded RXR) show that the liganded receptors adopt a more tight or compact structure compared to unliganded receptors, thus providing a structural basis to this modest resistance to general proteases [Renaud et al., 1995]. Based on these previous data and the data in the present manuscript, we hypothesize that liganded VDR and other nuclear receptors are indeed more resistant to proteolysis than unliganded receptors. Thus, specialized, and perhaps regulated, mechanisms may be in place to ensure efficient degradation and turnover of liganded receptors as a means to downregulate their activity in the nucleus. The interaction of liganded receptors with SUG1 via the AF-2 domain may, at least in part, target the liganded nuclear receptors to the proteosome machinery, where these more compact structures may be more efficiently degraded and inactivated. Although, the ubiquitinylated state of nuclear receptors is presently unknown, it is possible that their recognition by SUG1 within the 26S complex may provide a means to target proteins toward proteasome-mediated degradation.

Finally, observations detailed here may be applied to several other nuclear receptor systems. SUG1 interacts with a number of other receptors, including thyroid hormone receptors, retinoic acid receptors, and estrogen receptors [vom Bauer et al., 1996]. Interestingly, increases in the steady-state level of the estrogen receptor were also observed in ROS 17/2.8 cells following treatment with MG132, indicating that similar processes may be involved in estrogen receptor turnover [H. Masuyama and P.N. MacDonald, unpublished data]. Thus, the interaction of SUG1 with the AF-2 domain of various nuclear receptors may represent a general mechanism to regulate the duration and/or amplitude of any given steroid hormone response in a particular cell.

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