# Human Sug1/p45 is involved in the proteasome-dependent degradation of Sp1

Kaihong SU*, Xiaoyong YANG*, Mark D. ROOS*, Andrew J. PATERSON† and Jeffrey E. KUDLOW*†[1]

*Department of Cell Biology, University of Alabama at Birmingham, Birmingham, AL 35294, U.S.A., and †Department of Medicine, Division of Endocrinology and Metabolism, University of Alabama at Birmingham, Birmingham, AL 35294, U.S.A.

The transcription factor Sp1 was previously shown to undergo proteasome-dependent degradation when cells were glucose-starved and stimulated with the adenylate cyclase inducer, forskolin. However, the control of the Sp1 degradation process is largely unknown. Using *in vitro* and *in vivo* interaction studies, we show in the present study that Sp1 interacts with human Sug1 [hSug1, also known as p45 or thyroid-hormone-receptor interacting protein ('TRIP1')], an ATPase subunit of the 26 S proteasome and a putative transcriptional modulator. This interaction with Sp1 occurs through the C-terminus of hSug1, the region that contains the conserved ATPase domain in this protein. Both *in vitro* studies, in reconstituted degradation assays, and *in vivo* experiments, in which hSug1 is overexpressed in normal rat kidney cells, show that full-length hSug1 is able to stimulate the proteasome-dependent degradation of Sp1. However, hSug1 truncations that lack either the N- or C-terminal domain of hSug1 act as dominant negatives, inhibiting Sp1 degradation *in vitro*. Also, an ATPase mutant of hSug1, while still able to bind Sp1, acts as a dominant negative, blocking Sp1 degradation both *in vitro* and *in vivo*. These results demonstrate that hSug1 is involved in the degradation of Sp1 and that ATP hydrolysis by hSug1 is necessary for this process. Our findings indicate that hSug1 is an exchangeable proteasomal component that plays a critical regulatory role in the proteasome-dependent degradation of Sp1. However, hSug1 is not the factor limiting Sp1 degradation in the cells treated with glucosamine. This and other considerations suggest that hSug1 co-operation with other molecules is necessary to target Sp1 for proteasome degradation.

Key words: diabetes, glucosamine, glycosylation, O-GlcNAc, transcription.

## INTRODUCTION

The transcription factor Sp1 is important for the expression of many cellular genes, particularly housekeeping genes [1]. Although Sp1 has generally been considered to constitutively regulate gene expression, its activity and cellular content have been shown to be regulated during a variety of cellular processes [2,3]. We have previously shown that Sp1 is rapidly degraded by the proteasome in cells that are both glucose-starved and stimulated with cAMP [4]. Under these conditions, protein modification of Sp1 and other cellular proteins by O-linked *N*-acetylglucosamine (O-GlcNAc) is markedly reduced. Conversely, treatment of cells with glucose or glucosamine results in increased levels of O-GlcNAc and blocks the cAMP-mediated degradation of Sp1. We have postulated that Sp1 degradation by the proteasome might be part of the cellular response to nutrient deprivation and stress. The loss of Sp1 would down-regulate general gene transcription to conserve nutrients.

The 26 S proteasome is a complex organelle that degrades certain proteins via an ATP-dependent proteolytic pathway [5–7]. There is considerable evidence that the proteasome plays an important role in the regulation of diverse cellular processes, such as gene transcription, cell cycle progression and metabolism [5]. The 26 S complex is composed of a 20 S proteolytically active core and a 19 S regulatory complex [8–10]. The 19 S complex is located at the end(s) of the 20 S core and provides the components necessary for selective degradation of proteins and regulation of the activity of 20 S proteases [11,12]. The ATP-dependence for proteolysis by the 26 S proteasome derives from the ATPase subunits in the 19 S complex. Thus far at least six distinct, but related, ATPases have been found in the 19 S complex of mammalian proteasomes: human Sug1 [hSug1, also known as p45 or thyroid-hormone-receptor interacting protein ('TRIP1')], S4 (also known as p56), MSS1, TBP1, TBP7, and p42 [13–18]. It is thought that ATP hydrolysis by these ATPases provides energy for the unfolding and translocation of the substrates to the lumen of the 20 S core and for the assembly of the 26 S proteasome [19]. It is not clear why proteasomes have multiple ATPases and whether there is functional specificity among them. However, in yeast, it has been shown that different proteasomal ATPases have well-differentiated functions [20,21], and mutations in individual ATPases can markedly reduce the degradation of some substrates, while not affecting others [22]. Thus the ATPases in the proteasome might participate in the recognition of distinct substrates [5].

hSug1, one of the six known mammalian proteasomal ATPases, is the human homologue of yeast Sug1 [23]. Sug1 has been identified as an integral component of the 26 S proteasome in that it co-purifies with the proteasome in both conventional and nickel-chelate affinity chromatography [18]. In yeast, mutations in Sug1 cause the accumulation of mitotic cyclins and cell cycle arrest in G2/metaphase [22]. The processing of nuclear factor-$\kappa$B via the ubiquitin–proteasome pathway is inhibited when this protein is expressed in yeast with Sug1 mutations [24].

Abbreviations used: AAA, ATPase associated with a variety of cellular activities; C.Sug1, C-terminal domain of human Sug1; DMEM, Dulbecco's modified Eagle's medium; DTT, dithiothreitol; FBS, fetal bovine serum; GST, glutathione S-transferase; hSug1, human Sug1; hSug1mk, ATPase mutant of hSug1; LLnL, *N*-acetyl-Leu-Leu-norLeu-al; N.Sug1, N-terminal domain of hSug1; NRK, normal rat kidney; O-GlcNAc, O-linked *N*-acetylglucosamine; RT, reverse transcription; Stat3, signal transducer and activator of transcription 3.

[1] To whom correspondence should be addressed (e-mail kudlow@endo.dom.uab.edu).

Besides being a proteasome component, Sug1 has also been shown to interact with transcription factors and to be involved in the regulation of transcription. A yeast two-hybrid screen indicated that transcription factor c-Fos, also a known proteasome substrate, interacts with mammalian Sug1 [25]. Sug1 has been reported to mediate the association of transcriptional activators with the RNA polymerase II holoenzyme and to interact with the family members of nuclear receptors [26–29]. Recent evidence that Sug1 is a DNA helicase and that its ATPase activity is stimulated by RNAs supports the idea that Sug1 is involved in gene transcription [30,31]. How the activities of Sug1 as a transcriptional modulator and as a proteasome component can be reconciled is not clear. The observation that Sug1 is predominantly located in the nucleus implies a possible role for Sug1 in the degradation of transcription factors [25,32].

In the present study we report that hSug1 interacts with Sp1 protein both *in vitro* and *in vivo*. The C-terminal domain (amino acids 173–405) of hSug1 (C.Sug1) confers this interaction. Using an *in vitro* reconstituted degradation assay and an *in vivo* expression system, we show that wild-type hSug1 accelerates the proteasome degradation of Sp1, while inactivated mutant forms of hSug1 inhibit this Sp1 degradation. Our results provide direct evidence that hSug1 is involved in the degradation of at least one transcription factor, Sp1.

## MATERIALS AND METHODS

### Cell culture

Normal rat kidney (NRK) cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% (v/v) fetal bovine serum (FBS) with non-essential amino acids (Gibco BRL, Grand Island, NY, U.S.A.), 100 $\mu$g/ml penicillin (Sigma, St. Louis, MO, U.S.A.) and 50 $\mu$g/ml gentamicin (Sigma), at 37 °C in air/$CO_2$ (93:7). For stimulation of the cells, exponentially growing cells were seeded at $2.5 \times 10^6$ cells/100 mm diameter dish. After overnight incubation, the cell culture medium was changed to glucose-free DMEM containing 10% FBS, and the cells were incubated for an additional 20 h. The cells were then stimulated with vehicle alone, 100 $\mu$M forskolin or 5 mM glucosamine (Sigma) in glucose-free DMEM containing 10% FBS, and incubation was continued for an additional 24 h before cells were harvested.

### DNA constructs

The hSug1 cDNA was cloned and amplified by reverse-transcription (RT)-PCR from human breast cancer cell line MDA 468 mRNA using high-fidelity *Pfu* DNA polymerase (Stratagene, La Jolla, CA, U.S.A.). The PCR primers used to clone hSug1 cDNA were designed according to the hSug1 cDNA sequence. They were: 5′-primer, 5′-CGCGGATCCCCGATGGCGCTTGACGGACC-3′ and 3′-primer, 5′-CATAGGCCTTCACTTCCATAATTTCTTGATGG-3′. The N-terminal domain (amino acids 1–176) of hSug1 (N.Sug1) cDNA and C.Sug1 cDNA were cloned and amplified using the same strategy as that used for hSug1 full-length cDNA. The PCR primers used for N.Sug1 were: 5′-primer, 5′-CATGAATTCATGGCGCTTGACGGACCAG-3′ and 3′-primer, 5′-ATGCTCTAGACTTCGAAGAGCTCAGGATGC-3′. The PCR primers used for C.Sug1 were: 5′-primer, 5′-ATGCTCTAGAGAGCTCTTCGAAGCACTGG-3′ and the 3′-primer was the same as that used for cloning the full-length hSug1. The lysine residue at position 196 in hSug1 was converted to a methionine by site-directed mutagenesis to generate an ATPase mutant of hSug1 (hSug1mk). All the above constructs were confirmed by DNA sequencing.

### Expression and purification of glutathione S-transferase (GST) fusion proteins in *Escherichia coli* and vaccinia virus

The GST fusion proteins were expressed in *E. coli* and purified on glutathione–Sepharose beads (Pharmacia, Piscataway, NJ, U.S.A.) according to Pharmacia's pGEX protocol. The GST fusion proteins expressed using recombinant vaccinia viruses were purified as described previously [33].

### *In vitro* protein interaction assay

Equal quantities of the GST fusion proteins immobilized on glutathione–Sepharose affinity beads were washed extensively with binding buffer [50 mM $KH_2PO_4$ (pH 6.0), 100 mM KCl, 10 mM $MgCl_2$, 10% (v/v) glycerol and 1% (v/v) Tween-20]. The Promega *in vitro* transcription and translation ('TNT') kit from reticulocyte lysate was used as directed by the manufacturer to synthetically label Sp1 protein with [$^{35}$S]methionine. The labelled Sp1 (in 5 $\mu$l of reticulocyte lysate) was added to 5 $\mu$g of GST fusion proteins, immobilized on the glutathione beads. After incubation for 1 h at 4 °C the beads were washed extensively with the binding buffer and boiled in SDS/PAGE sample buffer. The proteins bound to the beads were then separated by SDS/PAGE. The $^{35}$S-labelled proteins that had bound to the beads were visualized by fluorography, while the unlabelled GST fusion proteins were visualized following Coomassie Brilliant Blue staining of the gel.

### The reconstituted degradation assay

Nuclear extract from differently treated NRK cells was prepared using the high salt extraction method described previously [33]. Vaccinia virus-expressed GST–hSug1, GST–hSug1mk, GST–N.Sug1, GST–C.Sug1 or GST were purified and eluted with 20 mM free glutathione. These GST proteins were preincubated with 50 $\mu$g of NRK nuclear extract at room temperature for 15 min in ATPase assay buffer containing 20 mM Tris/HCl (pH 7.5), 70 mM KCl, 1.5 mM $MgCl_2$ and 1.5 mM dithiothreitol (DTT). Vaccinia virus-expressed GST–Sp1 protein (10 ng) and 2 mM ATP were then added into the reaction mixture. Following incubation at 37 °C for 30 min, the reaction mixture was separated by SDS/PAGE and subjected to Western-blot analysis using anti-GST antibodies.

### *In vivo* protein interaction assay

NRK cells were cultured in 5 mM glucose medium to 90% confluency. The cells were infected with recombinant vaccinia viruses that allowed expression of the GST fusion proteins. After infection of NRK cells with recombinant viruses for 16–23 h, the cells were collected and resuspended in the whole-cell lysate buffer containing 20 mM Hepes (pH 7.9), 0.4 M NaCl, 3 mM $MgCl_2$, 0.5% Nonidet P40, 0.2 mM EDTA, 0.2 mM EGTA, 10% glycerol, 1 mM DTT, 1 mM PMSF, 5 $\mu$g/ml leupeptin and 5 $\mu$g/ml aprotinin. After incubation on ice for 10 min the cells were gently vortex-mixed for 10 s and centrifuged for 15 min at 16000 ***g*** at 4 °C. The supernatant was collected and designated as whole-cell lysate. Glutathione–Sepharose affinity beads were incubated with the whole-cell lysate for 2 h at 4 °C in the same buffer as above except that the concentration of NaCl was 150 mM. The beads were then collected and washed 4 times with the incubation buffer. The proteins bound to the beads were then separated by SDS/PAGE followed by Western-blot analysis.

## RESULTS

### Protein structure of hSug1 and the expression of hSug1 in *E. coli* and vaccinia virus

Structural analysis has identified hSug1 as a member of the AAA (ATPase associated with a variety of cellular activities) superfamily of putative ATPases [34]. As shown in Figure 1(A) the C-terminus of hSug1 contains a highly conserved ATPase domain ('CAD' or AAA domain). The N-terminus of hSug1 contains a putative coiled-coil structure whose function is still unknown. Recent evidence has shown that yeast and mammalian Sug1 are ATPases, and mutation of the lysine residue at position 196 eliminates their ATPase activity [30]. The lower panel in Figure 1(A) is a diagram showing the N-terminal domain (amino acids 1–176) and the C-terminal domain (amino acids 173–406) of hSug1 used in the present study. We cloned the cDNA of hSug1 by RT-PCR and expressed hSug1 as a GST fusion protein. As shown in Figure 1(B) GST–hSug1 was expressed with the correct molecular mass in mammalian cells using vaccinia virus (Figure 1B, lane 1) and in *E. coli* (Figure 1B, lane 2). GST expressed in *E. coli* is shown in Figure 1(B), lane 3.

### hSug1 protein interacts with Sp1 *in vitro*

As an initial step to investigate whether hSug1 is involved in the degradation of Sp1, we first examined the potential for direct interaction between the hSug1 protein and the Sp1 protein. We performed *in vitro* binding studies using a GST pull-down assay. GST and GST–hSug1 fusion proteins were expressed in *E. coli* (Figure 2A). Equal amounts of GST or GST–hSug1 were immobilized on glutathione–Sepharose beads and incubated with *in vitro*-translated, [$^{35}$S]methionine-labelled human Sp1 (full-length Sp1). As shown in Figure 2(B), GST–hSug1 pulled down Sp1 (lane 2), while GST alone did not pull down Sp1 (lane 1). We observed the same results with vaccinia virus-expressed GST fusion proteins (results not shown). These results show that hSug1 specifically interacts with Sp1 *in vitro*.

### C.Sug1 and hSug1mk interact with Sp1 efficiently *in vitro*

To determine which domain of hSug1 interacts with Sp1 we expressed C.Sug1 and N.Sug1 as GST fusion proteins in *E. coli* and tested their abilities to interact with Sp1 by *in vitro* protein-

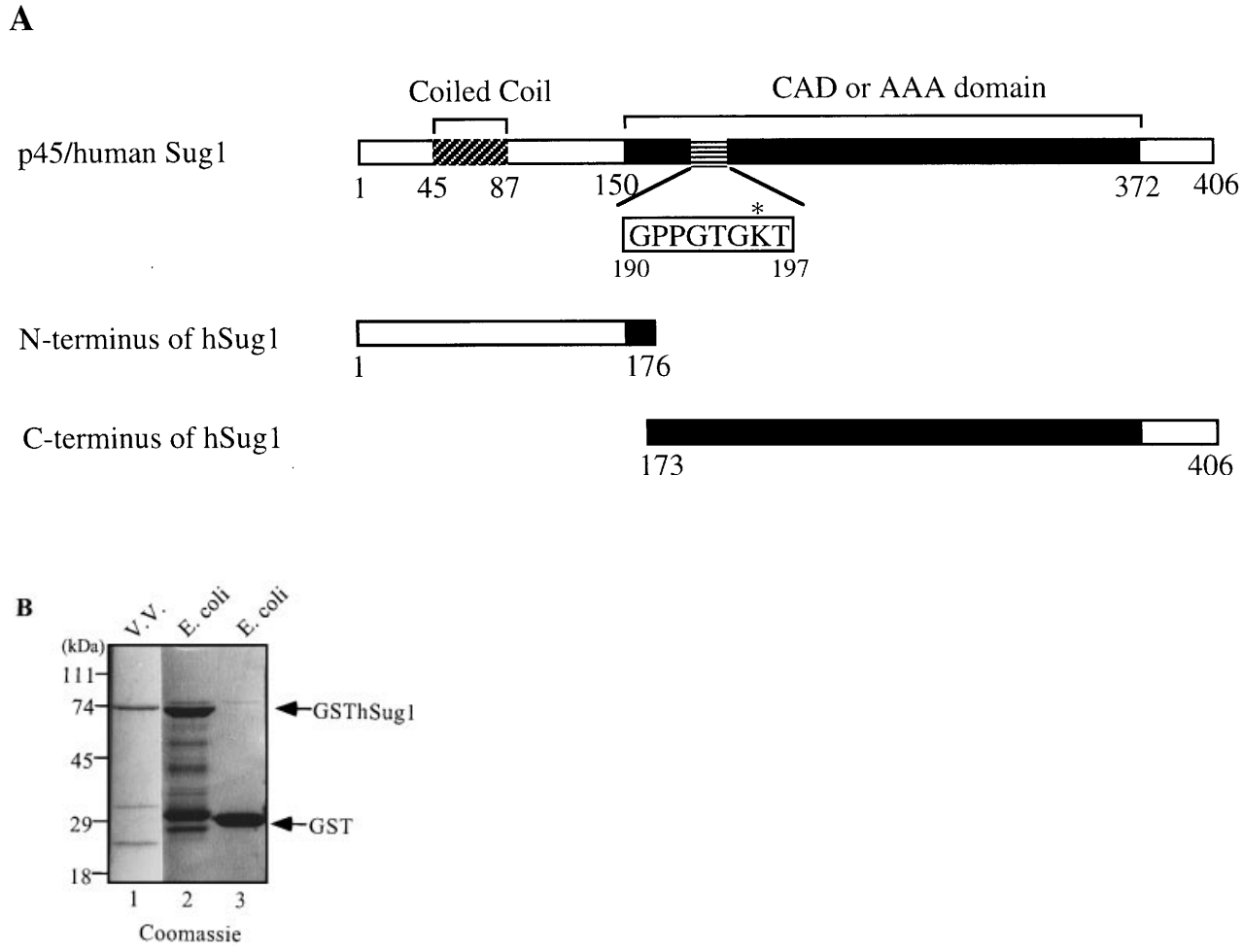


**Figure 1 hSug1 protein structure and expression of hSug1 in *E. coli* and vaccinia virus**

(**A**) hSug1 protein structure. The putative coiled-coil structure and the conserved ATPase domain are represented. The consensus ATP-binding motif is shown in the box. The lysine residue which is critical for ATP binding is indicated by the asterisk. The N- and C-terminal domains of hSug1 used in the present study are also shown. (**B**) The expression of hSug1 as GST fusion protein in *E. coli* and vaccinia virus (V.V.).

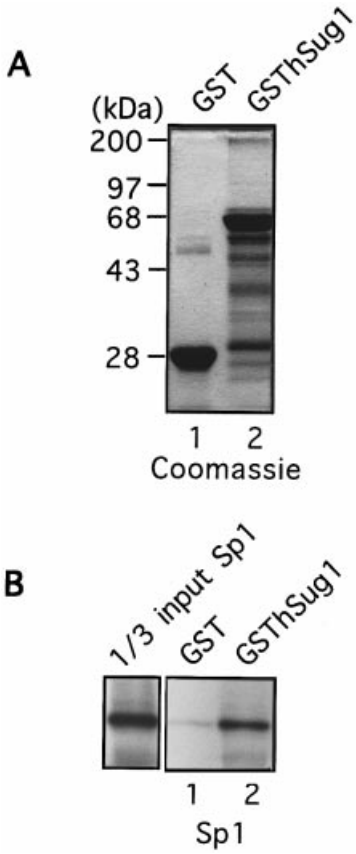


**Figure 2 hSug1 protein interacts with Sp1 *in vitro***

*In vitro*-translated, [$^{35}$S]methionine-labelled human Sp1 (amino acids 1–778) was incubated with GST–hSug1 (amino acids 1–406 of hSug1) or GST protein immobilized on glutathione–Sepharose beads. After the beads were washed, bound proteins were eluted and resolved by SDS/PAGE. Coomassie Brilliant Blue staining of the gel confirmed equivalency of loading (**A**). Fluorography shows the bound $^{35}$S-labelled proteins (**B**). The signals from one-third of the labelled input proteins are indicated in the left panel of (**B**).

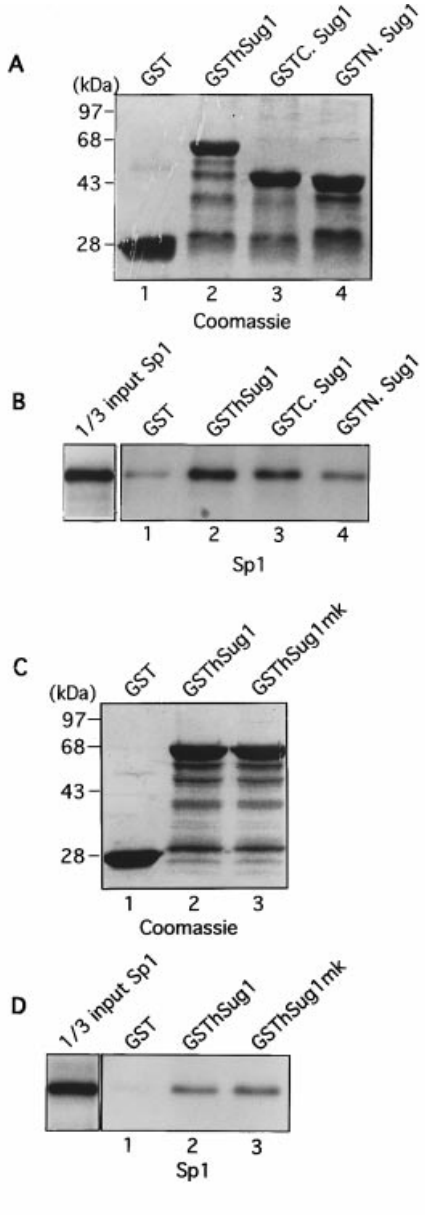


**Figure 3 C.hSug1 and hSug1mk interact with Sp1 efficiently *in vitro***

*In vitro*-translated [$^{35}$S]methionine-labelled human Sp1 was incubated with GST–hSug1 (amino acids 1–406 of hSug1), GST–C.hSug1 (amino acids 173–406), GST–N.hSug1 (amino acids 1–176), GST–hSug1mk (ATPase mutant) or GST immobilized on glutathione–Sepharose beads. After the beads were washed, bound proteins were eluted and resolved by SDS/PAGE. Coomassie Brilliant Blue staining of the gel confirmed equivalency of loading (**A** and **C**). Fluorography shows the bound $^{35}$S-labelled proteins (**B** and **D**). The signals from one-third of the labelled input proteins are indicated in the left panels of (**B**) and (**D**).

interaction studies. Equal amounts of GST, GST–hSug1 (full-length hSug1), GST–C.Sug1 and GST–N.Sug1 were applied in each reaction (Figure 3A). As shown in Figure 3(B) C.Sug1 bound Sp1 to approximately the same extent as the full-length hSug1 (lanes 2 and 3), while N.Sug1 bound Sp1 at a near background level, as indicated by the GST control (lanes 1 and 4). These results demonstrate that the C-terminal ATPase domain of hSug1 interacts with Sp1 efficiently *in vitro*.

The assembly of the 19 S complex and the 20 S catalytic core of the proteasome is ATP-dependent, suggesting that the interactions between the subunit proteins require ATP-binding [35]. To test if the ATPase activity of hSug1 is required for its interaction with Sp1, we used site-directed mutagenesis to replace lysine$^{196}$ with a methionine residue, thereby creating an ATPase mutant of hSug1, hSug1mk. We expressed hSug1mk as a GST fusion protein in *E. coli* and tested if this mutant hSug1 could interact with Sp1 using the *in vitro* interaction assay. Figure 3(C) shows that equal quantities of GST, GST–hSug1 or GST–hSug1mk proteins were applied in each reaction and Figure 3(D) shows that the mutant hSug1mk bound Sp1 to the same extent as the wild-type hSug1 (lanes 2 and 3). Our results show that the ATPase mutation of hSug1 does not detectably affect its binding affinity with Sp1.

### hSug1 interacts with Sp1 *in vivo*

To determine if the interaction of hSug1 and Sp1 can occur in living cells, we used vaccinia virus to express GST, GST–hSug1, GST–hSug1mk, GST–N.Sug1 and GST–C.hSug1 in NRK cells. The cells were cultured in 5 mM glucose medium in the presence of the proteasome inhibitor, *N*-acetyl-Leu-Leu-norLeu-al (LLnL), to prevent Sp1 degradation. The expressed GST fusion proteins were purified on glutathione–Sepharose beads and the endogenous Sp1 in the cells that co-purified with the indicated

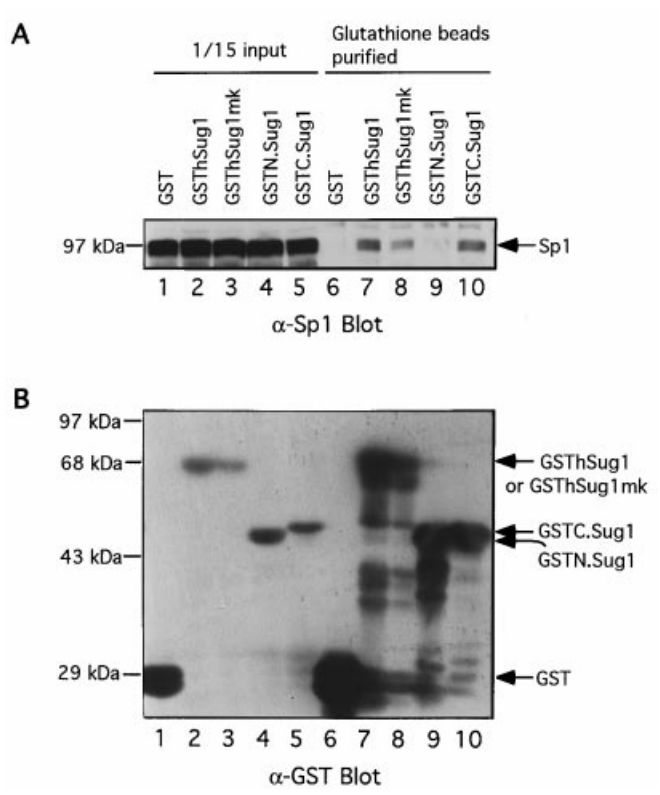


**Figure 4 hSug1 interacts with Sp1 *in vivo***

(**A**) NRK cells cultured in 5 mM glucose medium were infected with vaccinia virus expressing GST, GST–hSug1, GST–hSug1mk, GST–N.Sug1 or GST–C.Sug1 for 22 h in the presence of the proteasome inhibitor LLnL. The whole-cell lysate was prepared and the expressed GST fusion proteins were purified on glutathione–Sepharose beads. The glutathione beads were extensively washed and the proteins bound on the beads were separated by SDS/PAGE followed by Western-blot analysis using an anti-Sp1 antibody (lanes 6–10). Lanes 1–5 were loaded with the whole-cell lysate before glutathione bead purification. It represents one-fifteenth of the input whole-cell lysate for glutathione bead purification. (**B**) The membrane from (**A**) was stripped and reprobed with an anti-GST antibody.

GST fusion proteins was examined by Western-blot analysis using Sp1 antiserum. Figure 4(A) shows that the endogenous Sp1 did not co-purify with GST alone or with N.Sug1 (lanes 6 and 9), but did co-purify with the wild-type hSug1, hSug1mk and C.Sug1 (lanes 7, 8 and 10). The expression levels of the GST fusion proteins in the NRK cells are shown in Figure 4(B). Although the mutant hSug1mk appears to have pulled down less Sp1 than wild-type hSug1, the level of expression of GST–hSug1mk was less in this experiment than the level of expression of the wild-type form (Figure 4B, lanes 7 and 8). These results demonstrate that *in vivo*, hSug1 through its C-terminal domain can interact with Sp1 in an ATPase-independent fashion. These *in vivo* results are in general agreement with those of the *in vitro* studies.

### Wild-type hSug1 stimulates Sp1 degradation while hSug1mk, N.Sug1 and C.Sug1 inhibit Sp1 degradation *in vitro*

To determine if hSug1 is involved in the proteasome-dependent degradation of Sp1, we tested the effect of hSug1 proteins and its mutant forms on the degradation of Sp1 using an *in vitro* reconstituted assay. We have previously established this reconstituted degradation assay to map the domain in Sp1 that targets its proteasome-dependent degradation [33]. We have shown that recombinant GST–Sp1 protein is degraded *in vitro* in a proteasome-dependent manner in a two-step process by an activated nuclear extract made from glucose-starved and forskolin-stimulated NRK cells. Affinity-purified vaccinia virus-expressed GST–hSug1 was preincubated with the activated NRK nuclear extract. Then the substrate, GST–Sp1, and 2 mM ATP were added to the mixture and incubated at 37 °C for 30 min. The reaction mixture was separated by SDS/PAGE and subjected to Western-blot analysis using anti-GST antibodies. GST–Sp1 was not degraded when it was mixed with inactive nuclear extract from glucosamine-treated NRK cells (Figure 5, lane 1). GST–Sp1 was degraded when it was mixed with the activated nuclear extract from glucose-starved and forskolin-stimulated NRK cells and the usual degradation product, GST–SpX, was generated (lane 2). The addition of increasing amounts of GST–hSug1 protein resulted in further degradation of GST–Sp1, with the generation of more GST–SpX (Figure 5, lanes 3–5), while GST–hSug1 itself was not degraded. As a negative control GST alone had no effect on the GST–Sp1 degradation process (Figure 5, lanes 11 and 12). These results indicate that wild-type hSug1 augments the proteasome-dependent degradation of Sp1.

The ATPase subunits of the 26 S proteasome play important roles in the degradation of the substrates [36]. We therefore tested if the ATPase activity of hSug1 is required for Sp1 degradation. We performed the reconstituted degradation assay as described above. The addition of increasing amounts of GST–hSug1mk to activated extract resulted in progressively less GST–Sp1 degradation and less generation of GST–SpX (Figure 5, lanes 8–10). Thus not only is the ATPase activity of hSug1 necessary for its activity in the augmentation of Sp1 degradation, but also the ATPase mutant of hSug1 acts as a dominant negative, thereby inhibiting the proteasome-dependent processing of Sp1. Similarly, GST–N.Sug1 and GST–C.Sug1 also inhibited Sp1 degradation in a dose-dependent manner (Figure 5, lanes 13–18). In this experiment GST–hSug1mk, GST–N.Sug1 and GST–C.Sug1 themselves were not degraded. Taken together, while hSug1 stimulates Sp1 degradation in this *in vitro* system, both the N- and C-terminal domains of hSug1 are necessary for this function, and the ATPase activity of hSug1 is required in the degradation process of Sp1. The ability of mutant forms of Sug1 to act as dominant negatives and the ability of the wild-type form to augment processing suggest that hSug1 is at least one of the ATPases involved in the proteasome-dependent degradation of Sp1.

### Wild-type hSug1 stimulates Sp1 degradation, while hSug1mk inhibits Sp1 degradation, *in vivo*

To determine if the effects of hSug1 seen *in vitro* can occur *in vivo* on native Sp1, we again used vaccinia virus to drive expression of GST–hSug1 or GST–hSug1mk in NRK cells stressed with glucose starvation and forskolin stimulation. After glucose starvation NRK cells were stimulated with forskolin in glucose-free medium for 5 h, before the cells were infected with vaccinia virus. The cells were then incubated in glucose-free medium in the presence of forskolin for an additional 16 h before cells were harvested. The whole-cell lysate was prepared and the levels of endogenous Sp1 were examined by Western-blot analysis using an anti-Sp1 antibody. As shown in Figure 6(A) the content of endogenous Sp1 was lower in the cells expressing GST–hSug1 than that in the cells expressing GST alone (lanes 1 and 2), suggesting that the expression of wild-type hSug1 in stressed NRK cells further stimulated Sp1 degradation. However, the expression of hSug1mk did not affect the levels of Sp1 in this experiment (Figure 6A, lane 3). The same blot in Figure 6(A) was stripped and reprobed with anti-[signal transducer and activator

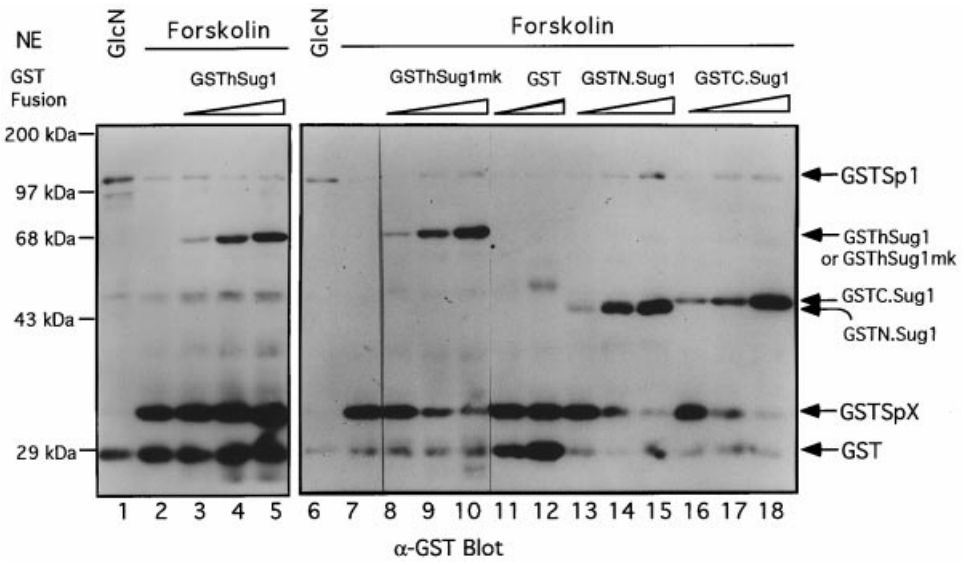


**Figure 5 Wild-type hSug1 stimulates Sp1 degradation while hSug1mk, N.Sug1 and C.Sug1 inhibit Sp1 degradation *in vitro***

Nuclear extract (NE) from the NRK cells that had been glucosamine-treated (GlcN) or glucose-starved and forskolin-treated was preincubated with purified proteins GST–hSug1, GST–hSug1mk, GST–N.hSug1, GST–C.hSug1 (for each fusion protein, approx. 4 ng, 12 ng and 24 ng of protein was used in the first, second and third doses respectively), GST (approximately 12 ng and 25 ng was used in the first and second doses, respectively) or buffer alone as a control. Recombinant GST–Sp1 protein was then added to the preincubation mixture. After incubation, the reaction mixture was resolved by SDS/PAGE and subjected to Western-blot analysis using an anti-GST antibody.

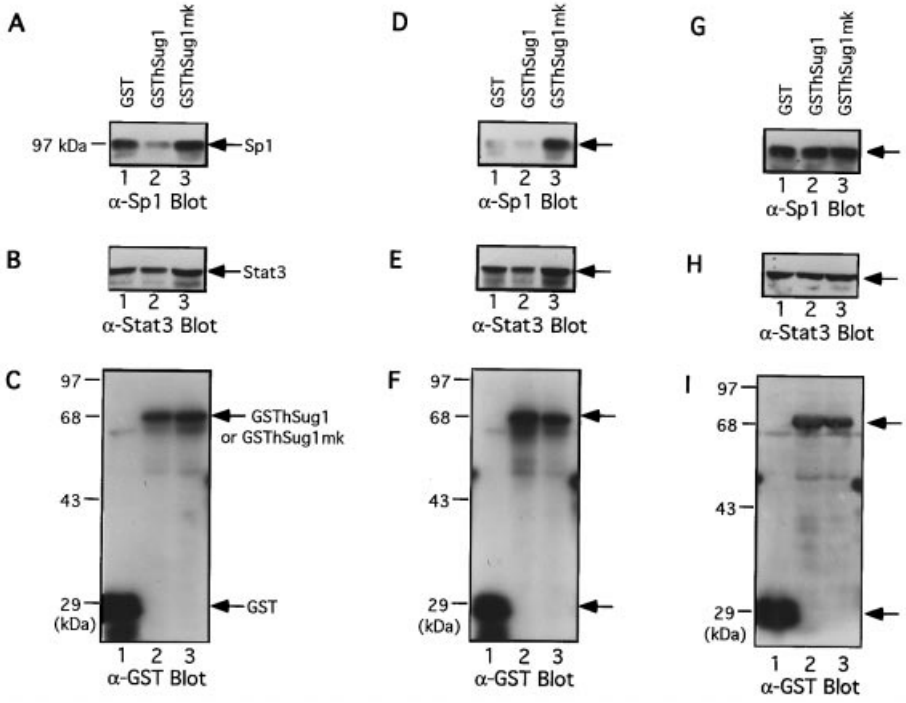


**Figure 6 Wild-type hSug1 stimulates Sp1 degradation while hSug1mk inhibits Sp1 degradation *in vivo***

After 24 h of glucose starvation NRK cells were stimulated with 100 $\mu$M forskolin (**A**–**F**) or 5 mM glucosamine (**G**–**I**) in glucose-free medium for 5 h. The cells were then infected with recombinant virus expressing GST, GST–hSug1 or GST–hSug1mk for 16 h (**A**–**C**) or 23 h (**D**–**F**) in glucose-free medium with 100 $\mu$M forskolin, or for 16 h in glucose-free medium with 5 mM glucosamine (**G**–**I**). The whole-cell lysate was prepared and separated by SDS/PAGE followed by Western-blot analysis using anti-Sp1 antibody (**A**, **D** and **G**). The membranes from (**A**), (**D**) and (**G**) were stripped and reprobed with an anti-Stat3 antibody as a protein loading control (**B**, **E** and **H**). The same membranes were stripped and reprobed with anti-GST antibody to show the levels of GST fusion proteins expressed in those cells (**C**, **F** and **I**).

of transcription 3 (Stat3)] antibody to confirm the known specificity of this proteasome response and to assess equal loading of proteins in each lane (Figure 6B). As shown in Figure 6(C), approximately similar amounts of GST fusion proteins were expressed in the cells.

In a separate experiment the NRK cells were incubated in glucose-free medium in the presence of forskolin for 23 h, rather than 16 h, after vaccinia virus infection. The cells were then harvested and lysed. In this case basal degradation of Sp1 was nearly complete in the cells expressing GST (Figure 6D, lane 1). In contrast, this degradation was effectively blocked in the cells expressing GST–hSug1mk (Figure 6D, lane 3). Since Sp1 degradation was nearly complete under the basal conditions of this experiment, the augmentation of degradation by wild-type hSug1 was not clearly appreciable (Figure 6D, lane 2). The specificity/loading control with Stat3 is shown in Figure 6(E) and the expression level of the GST fusion proteins is shown in Figure 6(F). Taken together these results suggest that the expression of hSug1 in stressed NRK cells is able to stimulate the degradation of endogenous Sp1, while the expression of hSug1mk is able to inhibit Sp1 degradation.

While nutritional stress stimulates the proteasome-dependent degradation of Sp1 in NRK cells, glucosamine treatment of these cells blocks this process. Since hSug1 augments Sp1 degradation in stressed cells, we next determined whether hSug1 could stimulate this degradation in glucosamine-treated cells. After glucose starvation, NRK cells were stimulated with glucosamine for 5 h before the cells were infected with the vaccinia viruses that drive expression of either GST–hSug1 or GST–hSug1mk. The cells were incubated in the presence of glucosamine for an additional 16 h before cells were harvested. The content of Sp1 in the whole-cell lysate was examined by Western-blot analysis using an anti-Sp1 antibody. The levels of Sp1 remained constant in the cells expressing GST, GST–hSug1 or GST–hSug1mk (Figure 6G, lanes 1–3). The protein loading (Figure 6H) and GST-fusion protein expression (Figure 6I) were confirmed as above. These results show that the expression of hSug1 cannot stimulate Sp1 degradation in glucosamine-treated cells. Similar results were obtained using the *in vitro* reconstituted degradation assay. The addition of GST–hSug1 protein to an extract from glucosamine-treated NRK cells was unable to restore the proteasome-dependent degradation of Sp1 (results not shown).

### hSug1 protein is not modified by O-GlcNAc

The inability of hSug1 to stimulate Sp1 degradation in glucosamine-treated cells could result from the modification of this protein by O-GlcNAc and an alteration in its ability to function in the degradation process. To determine if GST–hSug1 synthesized in glucosamine-treated NRK cells is modified by O-GlcNAc, we purified the protein from the cells and attempted to measure the O-GlcNAc content of the protein using the galactosyl-transferase method [37]. Recombinant proteins GST, GST–hSug1 and GST–SpE (SpE contains domain B, a glutamine-rich activation domain of Sp1 [37]) were expressed and purified from glucosamine or forskolin-treated cells as described above. Approximately 50 ng of these proteins was labelled with [$^3$H]galactose using galactosyltransferase. Our results show that GST–hSug1, prepared from either glucosamine- or forskolin-treated cells, was not labelled by [$^3$H]galactose, suggesting that GST–hSug1 was not modified by O-GlcNAc (results not shown). However, we readily detected the glycosylation of the Sp1 peptide, SpE, prepared from glucosamine-treated cells (results not shown). As a negative control, GST alone was not modified by O-GlcNAc (results not shown). We also performed Western-blot analysis using a GlcNAc-specific monoclonal antibody, RL-2, to attempt to detect O-GlcNAc modification of hSug1 under denaturing conditions [38]. Recombinant protein GST–hSug1 prepared from glucosamine-treated cells was not recognized by the RL-2 antibody (results not shown). Our results suggest that no O-GlcNAc modification of hSug1 protein is detectable under native or denaturing conditions.

## DISCUSSION

Previous studies have shown that Sp1 is rapidly degraded by the proteasome under the conditions of glucose-starvation and stimulation by cAMP [4]. However, the control of this degradation process is largely unknown. The accurate and specific selection of substrates for proteasome degradation may depend upon the signals in the substrates and also upon the proteasome itself. We have previously identified the N-terminal 54 amino acids of Sp1 as the major domain that targets Sp1 for proteasome-dependent degradation [33]. In the present study we further dissected the molecular basis for the control of Sp1 degradation.

Our results demonstrated that the proteasomal ATPase hSug1 is involved in the degradation of Sp1. Through its C-terminal domain, hSug1 interacts with Sp1 both *in vitro* and *in vivo*. While the interaction between these molecules does not depend on the ATPase activity of hSug1, in a reconstituted degradation assay and in the living cells, wild-type hSug1 stimulates Sp1 degradation, whereas the ATPase mutant hSug1mk and the N-and C-terminal domains of hSug1 block this Sp1 degradation. These results suggest that the recombinant hSug1 can exchange with the endogenous hSug1 in the degradation assay. Furthermore, full-length hSug1 with intact ATPase activity is both sufficient and necessary for the proteasome degradation of Sp1 under the experimental conditions. Like the ATPase mutant, the N- and C-terminal fragments of hSug1 both block Sp1 degradation possibly by perturbing the function of endogenous wild-type hSug1. There are two possible mechanisms to account for the observation that the N-terminal fragment of hSug1 inhibits full-length hSug1 function. The N-terminal Sug1 moiety encompasses a putative coiled-coil structure, which has been proposed to be involved in protein–protein interactions. Furthermore, previous evidence indicates that mammalian Sug1 can dimerize or oligomerize and that the N-terminus is required for Sug1 oligomerization [26]. If the activity of hSug1 depends on dimer formation, then the N-terminal hSug1 fragment could compete for dimerization with the full-length hSug1 and act as a dominant negative, thereby inhibiting the activity of this molecule in the Sp1 degradation process. The other possibility relates to the mechanism by which Sug1 is recruited to the 19 S complex of the proteasome. It has been determined that the coiled-coils in the N-terminal region of proteasomal ATPases probably direct the placement of these proteins within the proteasome [39]. Thus the truncated hSug1 N-terminal domain could inhibit the recruitment of full-length hSug1 to the proteasome by occupying the available docking sites on this organelle. Similarly the C-terminal fragment of hSug1 could compete with wild-type hSug1 for interaction with Sp1. Failure of the C-terminal fragment to dimerize or interact with the proteasome could account for the inhibitory properties of this Sug1 fragment on Sp1 degradation by the proteasome. Since there is high sequence similarity among the six proteasomal ATPases [34], our evidence does not exclude the possibility that other proteasomal ATPases may also be involved in the degradation of Sp1. However, the fact that the N-terminal domain of hSug1 has only about 18 % sequence similarity with other family members and yet inhibits Sp1 degradation argues that hSug1 is

the predominant proteasomal ATPase responsible for Sp1 degradation.

Regulation of transcription factor abundance by proteasome-dependent degradation is important in the control of transcription and has been shown to play a role in the regulation of transcription factors that include c-Fos, c-Jun and p53 [40–42]. However, the controlling mechanism for the proteasome recognition of transcription factors and the proteins involved in this recognition are not totally clear. Among the six ATPases in the 26 S proteasome Sug1 has been most extensively shown to interact with transcription factors, although the functional role of this interaction has not been definitively elucidated. An intriguing possibility is that hSug1 might co-operate in the targeting of certain types of transcription factors for proteasome degradation. There is precedence for this idea in *E. coli*. The 26 S proteasome resembles the *E. coli* protease ClpAP (also known as Ti) in its general organization and structure [5]. The ClpP protease cylinder is capped at both ends by a large regulatory ATPase complex, ClpA/ClpX. Interestingly, it has been shown that the ATPases ClpA and ClpX direct the substrate specificity for the ClpP protease to different proteins [43–45]. In yeast, mutations in the different regulatory ATPases of the proteasomes result in different phenotypes [21] and alter the degradation of different substrates [46]. This result has suggested that the ATPases co-operate in the degradation of individual substrates [21]. Similarly, the different ATPases in the mammalian 26 S proteasome may direct substrate recognition thereby providing an additional means of control of substrate specificity for the proteasome. There is some evidence supporting this idea. Human proteasomal S6 ATPase (and MS73 ATPase in *Manduca Sexta*) binds the ankyrin repeats present in a set of proteasome substrates and this interaction may complement ubiquitination as recognition signal by the 26 S proteasome [47]. For Sug1 it has been shown that this proteasomal ATPase is predominantly located in the nucleus, while the proteasome is distributed in both the cytoplasm and the nucleus [32,48]. Sug1 interacts with c-Fos [25] and also appears to be involved in the degradation of the vitamin D nuclear receptor [49]. The present study clearly shows that hSug1 is also required for the proteasomal degradation of another transcription factor, Sp1. However, whether hSug1 participates in the recognition of certain types of transcription factors by the proteasome and whether there is a common structural motif in these proteins recognized by hSug1 remains to be elucidated.

Previously we have identified an N-terminal domain of Sp1 that targets its proteasome-dependent degradation. Elimination of this target domain impairs Sp1 degradation and stabilizes the protein. Our preliminary results indicate that hSug1 does not interact with this target domain of Sp1 (results not shown). We have also shown that increased modification of proteins by O-GlcNAc is associated with Sp1 stabilization [4] and that nuclear extract from glucosamine-treated cells fails to degrade recombinant Sp1 [33]. However, hSug1 is not modified by O-GlcNAc even in cells that had been treated with glucosamine, and hSug1 cannot restore the activity of the proteasomes derived from glucosamine-treated cells. This evidence suggests that hSug1 is not the unique factor that links the O-GlcNAc state of the cell with Sp1 stability and targets Sp1 for degradation. Rather, it appears that Sp1 degradation results from the co-operation of other proteasome factors that require Sug1. We propose that this other factor directly recognizes the N-terminal target of Sp1 in an O-GlcNAc-dependent fashion and co-operates with Sug1 in the proteasomal degradation process. Collectively our results show that hSug1 interacts with Sp1 through a region in the C-terminal half of hSug1 and this interaction is required for the proteasome-dependent degradation of Sp1. These findings provide more detail concerning the molecular interactions involved in the proteasomal degradation of Sp1 and shed light on the function of hSug1 as a proteasomal component.

This work was supported by a program grant from the Juvenile Diabetes Foundation International.

## REFERENCES


1 Kadonaga, J. T., Jones, K. A. and Tjian, R. (1986) Promoter specific activation of RNA polymerase II by Sp1. Trends Biochem. Sci. **11**, 20–23

2 Mortensen, E. R., Marks, P. A., Shiotani, A. and Merchant, J. L. (1997) Epidermal Growth Factor and Okadaic Acid Stimulate Sp1 Proteolysis. J. Biol. Chem. **272**, 16540–16547

3 Shin, T. H., Paterson, A. J., Grant, III, J. H., Meluch, A. A. and Kudlow, J. E. (1992) 5-Azacytidine Treatment of HA-A Melanoma Cells Induces Sp1 Activity and Concomitant Transforming Growth Factor a Expression. Mol. Cell. Biol. **12**, 3998–4006

4 Han, I. and Kudlow, J. E. (1997) Reduced O Glycosylation of Sp1 Is Associated with Increased Proteasome Susceptibility. Mol. Cell. Biol. **17**, 2550–2558

5 Coux, O., Tanaka, K. and Goldberg, A. L. (1996) Structure and Functions of the 20S and 26S Proteasomes. Annu. Rev. Biochem. **65**, 801–847

6 Driscoll, J. and Goldberg, A. L. (1990) The Proteasome (Multicatalytic Protease) Is a Component of the 1500-kDa Proteolytic Complex Which Degrades Ubiquitin-conjugated Proteins. J. Biol. Chem. **265**, 4789–4792

7 Laney, J. D. and Hochstrasser, M. (1999) Substrate targeting in the ubiquitin system. Cell **97**, 246–251

8 Arrigo, A. P., Tanaka, K., Goldberg, A. L. and Welch, W. J. (1988) Identity of the 19S 'prosome' particle with the large multifunctional protease complex of mammalian cells (the proteasome). Nature (London) **331**, 192–194

9 Larsen, C. N. and Finley, D. (1997) Protein translocation channels in the proteasome and other proteases. Cell **91**, 431–434

10 Tanaka, K., Yoshimura, T., Kumatori, A., Ichihara, A., Ikai, A. and Nishigai, M. (1988) Proteasomes (Multi-protease Complexes) as 20S Ring-Shaped Particles in a Variety of Eukaryotic Cells. J. Biol. Chem. **263**, 16209–16217

11 Deveraux, Q., Ustrell, V., Pickart, C. and Rechsteiner, M. (1994) A 26S protease subunit that binds ubiquitin conjugates. J. Biol. Chem. **269**, 7059–7061

12 van Nocker, S., Deveraux, Q., Rechsteiner, M. and Vierstra, R. D. (1996) Arabidopsis MBP1 gene encodes a conserved ubiquitin recognition component of the 26S proteasome. Proc. Natl. Acad. Sci. U.S.A. **93**, 856–860

13 DeMartino, G. N., Moomaw, C. R., Zagnitko, O. P., Proske, R. J., Ma, C. P., Afendis, S. J., Swaffield, J. C. and Slaughter, C. A. (1994) PA700, an ATP-dependent activator of the 20S proteasome, is an ATPase containing multiple members of a nucleotide binding protein family. J. Biol. Chem. **269**, 20878–20884

14 Dubiel, W., Ferrell, K., Pratt, G. and Rechsteiner, M. (1992) Subunit 4 of the 26S protease is a member of a novel eukaryotic ATPase family. J. Biol. Chem. **267**, 22699–22702

15 Dubiel, W., Ferrell, K. and Rechsteiner, M. (1993) Peptide sequencing identifies MSS1, a modulator of HIV Tat-mediated transactivation, as subunit 7 of the 26S proteasome. FEBS Lett. **323**, 276–278

16 Glickman, M. H., Rubin, D. M., Fried, V. A. and Finley, D. (1998) The regulatory particle of the *S. cerevisiae* proteasome. Mol. Cell. Biol. **18**, 3149–3162

17 Richmond, C., Gorbea, C. and Rechsteiner, M. (1997) Specific interactions between ATPase subunits of the 26S proteasome. J. Biol. Chem. **272**, 13403–13411

18 Rubin, D. M., Coux, O., Wefes, I., Hengartner, C., Young, R. A., Goldberg, A. L. and Finley, D. (1996) Identification of the gal4 suppressor Sug1 as a subunit of the yeast 26S proteasome. Nature (London) **379**, 655–657

19 Armon, T., Ganoth, D. and Hershko, A. (1990) Assembly of the 26S complex that degrades proteins ligated to ubiquitin is accompanied by the formation of ATPase activity. J. Biol. Chem. **265**, 20723–20726

20 Glickman, M. H., Rubin, D. M., Fu, H., Larsen, C. N., Coux, O., Wefes, I., Pfeifer, G., Cjeka, Z., Vierstra, R., Baumeister, W. et al. (1999) Function analysis of the proteasome regulatory particle. Mol. Biol. Rep. **26**, 21–28

21 Rubin, D. M., Glickman, M. H., Larsen, C. N., Dhruvakumar, S. and Finley, D. (1998) Active site mutants in the six regulatory particle ATPases reveal multiple roles for ATP in the proteasome. EMBO J. **17**, 4909–4919

22 Ghislain, M., Udvardy, A. and Mann, C. (1993) S. cerevisiae 26S protease mutants arrest cell division in G2/metaphase. Nature (London) **366**, 358–362

23 Akiyama, K., Yokota, K., Kagawa, S., Shimbara, N., DeMartino, G. N., Slaughter, C. A., Noda, C. and Tanaka, K. (1995) cDNA cloning of a new putative ATPase subunit p45 of the human 26S proteasome, a homolog of yeast transcription factor Sug1p. FEBS Lett. **363**, 151–156

24 Sears, C., Olesen, J., Rubin, D., Finley, D. and Maniatis, T. (1998) NF-kB p105 Processing via the Ubiquitin-Proteasome Pathway. J. Biol. Chem. **273**, 1409–1419

25 Wang, W., Chevray, P. M. and Nathans, D. (1996) Mammalian Sug1 and c-Fos in the nuclear 26S proteasome. Proc. Natl. Acad. Sci. U.S.A. **93**, 8236–8240

26 Baur, E. V., Zechel, C., Heery, D., Heine, M. J. S., Garnier, J. M., Vivat, V., Douarin, B. L., Gronemeyer, H., Chambon, P. and Losson, R. (1996) Differential ligand-dependent interactions between the AF-2 activating domain of nuclear receptors and the putative transcriptional intermediary factors mSUG1 and TIF1. EMBO J. **15**, 110–124

27 Lee, J. W., Ryan, F., Swaffield, J. C., Johnston, S. A. and Moore, D. D. (1995) Interaction of thyroid-hormone receptor with a conserved transcriptional mediator. Nature (London) **374**, 91–94

28 Swaffield, J. C., Melcher, K. and Johnston, S. A. (1995) A Highly conserved ATPase protein as a mediator between acidic activation domains and the TATA-binding protein. Nature (London) **374**, 88–91

29 Weeda, G., Rossignol, M., Fraser, R. A., Winkler, G. S., Vermeulen, W., Veer, L. J., Ma, L., Hoeijmakers, J. H. J. and Egly, J. M. (1997) The XPB subunit of repair/transcription factor TFIIH directly interacts with SUG1, a subunit of the 26S proteasome and putative transcription factor. Nucleic Acids Res. **25**, 2274–2283

30 Fraser, R. A., Rossignol, M., Heard, D. J., Egly, J.-M. and Chambon, P. M. (1997) SUG1, a putative transcriptional mediator and subunit of the PA 700 proteasome regulatory complex, is a DNA helicase. J. Biol. Chem. **272**, 7122–7126

31 Makino, Y., Yamano, K., Kanemaki, M., Morikawa, K., Kishimoto, T., Shimbara, N., Tanaka, K. and Tamura, T. (1997) SUG1, a component of the 26S Proteasome, Is an ATPase Stimulated by Specific RNAs. J. Biol. Chem. **272**, 23201–23205

32 Russell, S. J., Steger, K. A. and Johnston, S. A. (1999) Subcellular localization, stoichiometry, and protein levels of 26S proteasome subunits in yeast. J. Biol. Chem. **274**, 21943–21952

33 Su, K., Roos, M. D., Yang, X., Han, I., Paterson, A. J. and Kudlow, J. E. (1999) An N-terminal region of Sp1 targets its proteasome-dependent degradation in vitro. J. Biol. Chem. **274**, 15194–15202

34 Makino, Y., Yogosawa, S., Kanemaki, M., Yoshida, T., Yamano, K., Kishimoto, T., Moncollin, V., Egly, J., Maramatsu, M. and Tamura, T. (1996) Structures of the Rat Proteasomal ATPases: Determination of Highly Conserved Structural Motifs and Rules for Their Spacing. Biochem. Biophys. Res. Commun. **220**, 1049–1054

35 Orino, E., Tanaka, K., Tamura, T., Sone, S., Ogura, T. and Ichihara, A. (1991) ATP-dependent reversible association of proteasomes with multiple protein components to form 26S complexes that degrade ubiquitinated proteins in human HL-60 cells. FEBS Lett. **284**, 206–210

36 Hoffman, L. and Rechsteiner, M. (1994) Activation of the multicatalytic protease. J. Biol. Chem. **269**, 16890–16895


Received 5 January 2000/29 February 2000; accepted 23 March 2000


37 Roos, M. D., Su, K., Baker, J. R. and Kudlow, J. E. (1997) O Glycosylation of an Sp1-Derived Peptide Blocks Known Sp1 Protein Interactions. Mol. Cell. Biol. **17**, 6472–6480

38 Sayeski, P. P. and Kudlow, J. E. (1996) Glucose Metabolism to Glucosamine Is Necessary for Glucose Stimulation of Transforming Growth Factor-a Gene Transcription. J. Biol. Chem. **271**, 15237–15243

39 Gorbea, C., Taillandier, D. and Rechsteiner, M. (1999) Assembly of the regulatory complex of the 26S proteasome. Mol. Biol. Rep. **26**, 15–19

40 Rolfe, M., Beer-Romero, P., Glass, S., Eckstein, J., Berdo, I., Theodoras, A., Pagano, M. and Draetta, G. (1995) Reconstitution of p53-ubiquitinylation reactions from purified components: the role of human ubiquitin-conjugating enzyme UBC4 and E6-associated protein (E6AP). Proc. Natl. Acad. Sci. U.S.A. **92**, 3264–3268

41 Stancovski, I., Gonen, H., Orian, A., Schwartz, A. L. and Ciechanover, A. (1995) Degradation of the Proto-Oncogene Product c-Fos by the Ubiquitin Proteolytic System In Vivo and In Vitro: Identification and Characterization of the Conjugating Enzymes. Mol. Cell. Biol. **15**, 7106–7116

42 Treier, M., Staszewski, L. M. and Bohmann, D. (1994) Ubiquitin-Dependent c-Jun Degradation In Vivo Is Mediated by the d Domain. Cell **78**, 787–798

43 Pak, M. and Wickner, S. (1997) Mechanism of protein remodeling by ClpA chaperone. Proc. Natl. Acad. Sci. U.S.A. **94**, 4901–4906

44 Schweder, T., Lee, K. H., Lomovskaya, O. and Matin, A. (1996) Regulation of Escherichia coli starvation sigma (sigma s) by ClpXP protease. J. Bacteriol. **178**, 470–476

45 Wawrzynow, A., Wojtkowiak, D., Marszalek, J., Banecki, B., Jonsen, M., Graves, B., Georgopoulos, C. and Zylicz, M. (1995) The ClpX heat-shock protein of Escherichia coli, the ATP-dependent substrate specificity component of the ClpP-ClpX protease, is a novel molecular chaperone. EMBO J. **14**, 1867–1877

46 Javerzat, J. P., McGurk, G., Cranston, G., Barreau, C., Bernard, P., Gordon, C. and Allshire, R. (1999) Defects in components of the proteasome enhance transcriptional silencing at fission yeast centromeres and impair chromosome segregation. Mol. Cell. Biol. **19**, 5155–5165

47 Dawson, S., Hastings, R., Takayanagi, K., Reynolds, S., Low, P., Billett, M. and Mayer, R. J. (1997) The 26S-proteasome: regulation and substrate recognition. Mol. Biol. Rep. **24**, 39–44

48 Peters, J. M., Franke, W. W. and Kleinschmidt, J. A. (1994) Distinct 19S and 20S subcomplexes of the 26S proteasome and their distribution in the nucleus and the cytoplasm. J. Biol. Chem. **269**, 7709–7718

49 Masuyama, H. and MacDonald, P. N. (1998) Proteasome-mediated degradation of the vitamin D receptor (VDR) and a putative role for SUG1 interaction with the AF2 domain of VDR. J. Cell. Biochem. **71**, 429–440