The ubiquitin-proteasome pathway is required for the function of the viral VP16 transcriptional activation domain

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Abstract The ability of the activation domain of specific protein factors to regulate transcription is intimately connected to their ubiquitin-mediated proteolysis. Here, we provide evidence that ubiquitin-proteasome function is required for a family of synthetic viral VP16 transcription activators in mammalian cells. Blocking the degradation of VP16 activators, through proteasome inhibitors or by disrupting the ubiquitylation function, severely compromises their transcriptional activity. Overexpression of SUG-1, a subunit of the proteasome, reduces both transactivation and degradation of VP16 activators. The inhibitory effect of SUG-1 overexpression is enhanced when a single non-removable ubiquitin moiety is fused to the amino-terminus of the VP16 activator. The 19S regulatory subunit of the proteasome physically associates with the general transcription factor TFIIH, indicating the direct involvement of the proteasome in transcription. These results support a model in which ubiquitin plays an accessory role, in recruiting the 19S regulatory subunit of the proteasome, for transcriptional activation.

Key words: Ubiquitylation; Proteasome; Protein degradation; Transcription; Viral VP16

1. Introduction

In eukaryotic cells, ubiquitin (Ub)-mediated proteolysis is a major pathway responsible for the destruction of short-lived transcription factors, including those implicated in the control of cell growth, signal transduction, programmed cell death and regulation of transcription [1]. The function and fate of transcription factors, e.g. Myc, p53, NF-κB and E2F1, are kept under tight regulatory control through a highly responsive and coordinated Ub-mediated proteolytic process within cells. The common feature of Ub-mediated proteolysis is that the highly conserved 76 amino acid Ub protein is covalently attached to the target proteins through a series of enzymes, namely E1 Ub-activating enzyme (UBA), E2 Ub-conjugating enzyme (UBC) and E3 Ub-ligase. E1 first activates Ub and transfers the activated Ub to E2. From the E2 enzyme, the Ub is transferred directly to the target protein or indirectly through an E3 Ub-protein ligase [2]. The polyubiquitylated protein is then recognized and degraded by the 26S proteasome, a large complex with multiple proteolytic activities [1,2].

The proteins targeted for destruction by Ub-mediated proteolysis usually contain an element that signals their ubiquitylation. These elements are often portable and are sometimes referred to as degrons [2]. The degrons have been found to overlap transcriptional activation domains (TADs) [3,4]. The extent of Ub-mediated proteasomal degradation has been shown to correlate with the potency of the TADs [3,5]. Such convergence of the transcriptional activation and the proteolytic signaling raises the possibility that the Ub-proteasome pathway could be involved in both transcriptional activation and protein degradation and these two events are somehow linked together, at least for some specific transcription factors. Recently, it has been reported in the yeast system that activator ubiquitylation is essential for transcriptional activation [3]. In addition, the 19S regulatory subunit of the 26S proteasome has been shown to be required for efficient transcription elongation by RNA polymerase II [6]. A 19S proteasome subcomplex was also shown to be recruited to activate the GAL1-10 promoter in yeast [7]. These observations underscore the potential link between Ub-mediated degradation and transcriptional activation.

In this report, we show that the Ub-mediated proteolytic process is involved in transcriptional activation mediated by a family of synthetic transcription activators derived from a reiterated eight amino acid sequence from the herpes simplex viral VP16 TAD. We further provide evidence that SUG-1, a subunit of the proteasome, is recruited for both transcription activation and the degradation of VP16 activators. The presented data also demonstrate that the 19S regulatory subunit of the proteasome is physically associated with the general transcription factor TFIIH. These observations reveal a critical role of ubiquitylation and the proteasome in coordinating protein degradation and transactivation during transcription.

2. Materials and methods

2.1. Plasmid constructs

The pCG-GAL4 expression constructs, encoding synthetic transcription activators containing the GAL4 DNA binding domain (BD), fused with one to six copies of VP16 VN8 modules or three copies of mutant VN8 sequence [3], were acquired from William P. Tansey. The GAL4 reporter construct pG5-*Luc*, containing five copies of the GAL4 binding sequence, was purchased from Promega. Mam-

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malian expression constructs with Ub moieties fused to the N-terminus of GAL4-VP16 were obtained upon cloning the Ub coding DNA generated by polymerase chain reaction (PCR) of yeast genomic DNA. The C-terminal glycine residue of the Ub primer sequence was changed to alanine. Expression vectors of Myc-tagged SUG-1 and SUG-1m, containing a lysine to methionine mutation at residue 196 (K196M), were constructed by routine PCR-based cloning methods. Bacterial expression constructs for glutathione *S*-transferase (GST) and its fusion proteins were constructed using the pGEX-4T-1 vector (Amersham Pharmacia Biotech). All recombinant DNA constructs were verified by sequencing.

2.2. Antibodies and biochemicals

Anti-HA (12CA5) and anti-Myc (9E10) antibodies and FuGENE 6 transfection reagents were from Roche Molecular Biochemicals. Monoclonal anti-GAL4 BD and anti-SUG-1 antibodies were purchased from BD Biosciences. Rabbit polyclonal antibodies against the proteasome 19S subunit S1 and TFIIH component XPB were respectively obtained from Affinity BioReagents and Santa Cruz Biotechnology. Proteasome inhibitors MG132 and lactacystin were from Calbiochem.

2.3. Cell culture, transient transfection, luciferase reporter assay and Western blotting analysis

Transient transfections were carried out in NHF (OSU2), LFS (MDAH041) and HeLa cells at 37°C or the mouse UBA temperature-sensitive ts85 cell line [8] at 32°C as described earlier [9–12]. The cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. About 18–20 h before transfection, cells were plated at a density of $1–3\times10^5$ per 35 mm diameter dish (or $3–10\times10^5$ per 100 mm diameter dish for Western analysis). The transfection was carried out with FuGENE 6 transfection reagent according to the manufacturer's recommendation. Approximately 12 h after transfection, the DNA–FuGENE mix was removed and cells were treated with proteasome inhibitor MG132 (1 and 5 μM) or its dimethylsulfoxide (DMSO) vehicle as a control for an additional 12 h period. The cells were harvested for luciferase assay and Western analysis. When needed, ts85 cells were plated at 5×10^6 per 100 mm dish, transfected for 24 h and the cells were divided into six 35 mm diameter dishes and transferred in triplicate to a restrictive temperature regime of 37°C or permissive 32°C for another 24 h. In some experiments, transfection periods were extended to 24 or 48 h and MG132 (10 μM) was added 10 h before harvesting. The results of relative luciferase units (RLU) of the enzyme activity assay were standardized against the total cellular protein and normalized to the values obtained in controls, or in the absence of MG132. All the transfection and reporter assay experiments were independently performed at least three times each and error bars represent the S.E.M. of triplicate data points. For Western blotting analysis, cells were lysed by boiling for 10 min in sample buffer (2% sodium dodecyl sulfate (SDS), 10% glycerol, 10 mM dithiothreitol, 62 mM Tris–HCl, pH 6.8, 10 μg/ml pepstatin and 10 μg/ml leupeptin) and protein concentration was determined by DC Bio-Rad assay. Western blotting was performed using appropriate antibodies and as previously described [13,14].

2.4. GST pull-down and immunoprecipitation

Whole cell extracts (WCE) were made from HeLa cells by the published procedures [15,16]. The WCE were dialyzed overnight against affinity column buffer (10 mM Tris–HCl, pH 7.9, 100 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, 10% glycerol) containing 1 mM phenylmethylsulfonyl fluoride and concentrated before GST pull-down, or GST-Ub-like (Ubl) affinity chromatography. GST pull-down experiments were performed in the affinity column buffer by incubating the HeLa WCE with the GST fusion proteins loaded on glutathione Sepharose-4B beads at 4°C for 1 h. The beads were washed and bound proteins were recovered by boiling in SDS loading buffer and analyzed by Western blotting. For immunoprecipitation of GST-Ubl affinity-purified proteins, the HeLa WCE were applied to a glutathione Sepharose-4B column previously loaded with GST-Ubl fusion proteins. After washing the column thoroughly, the bound proteins were eluted with 10 mM glutathione in 50 mM Tris–HCl (pH 8.0). The pooled eluates were concentrated using a 10 kDa cut-off Ultrafree-MC unit. The immunoprecipitation was performed in the affinity column buffer using 1 μg of either anti-XPB or normal rabbit IgG at 4°C for 1 h. Protein A/G agarose beads, previously equilibrated in column buffer containing 100 μg/ml bovine serum albumin, were added and the samples were incubated for an extra hour. The beads were washed four times with affinity column buffer and the bound proteins were eluted and analyzed by Western blotting using indicated antibodies.

3. Results

3.1. Close correlation between activation, degradation and MG132-mediated transcriptional inhibition

The convergence of the transcriptional activation and the proteolytic signaling raises the possibility that the Ub-proteasome pathway could be involved in both transcriptional activation and protein degradation. We have addressed this possibility using a series of synthetic transcriptional activators (Fig. 1A), derived by the reiteration of an eight amino acid sequence (DFDLDMLG, VN8) from the VP16 TAD. It may be noted that several lines of experimental observation have already described the functional features of these transcription activators. First, the potency of activators to conduct transcription correlates with their degradation. Second, the integrity of the TAD is essential for both their transactivation and degradation. A phenylalanine to alanine mutation (DADADMLG, mutant VN8) abolishes transcription, reduces affinity for transcription factor TFIID [17] and TFIIB [5] and also protects them from degradation [3,5]. Third, the potency of transcriptional activators correlates with their ubiquitylation status, the potent ones being more heavily ubiquitylated than the weaker ones [3]. So, we asked whether impairment of proteasome function by proteasome inhibitor could affect transcriptional activation. As shown in Fig. 1, augmented copies of VN8 modules increase the transcriptional potency (Fig. 1B,C) but decrease the steady-state levels of the activator molecules (Fig. 1E). The MG132 treatment causes a significant accumulation of unstable molecules, confirming that the functional proteasome is required for their degradation (Fig. 1E). More importantly, MG132 decreases the capacity of these activators to conduct effective transcriptional activation and severely inhibits the transcription mediated by the potent activators. MG132 treatment decreases the transcription mediated by GAL4-1×, -2×, -3× and -6× VN8 about 2-, 3-, 4- and 10-fold, respectively. It does not affect the transcription mediated by GAL4-3×M (three copies of mutant VN8), which has a decidedly reduced transcription potency. Thus, there is a close correlation between the relative inhibition (absence vs. presence of MG132) of transcriptional activation and the potency of the activators, except for the one with only the GAL4 DNA BD (Fig. 1B,C). On the other hand, the MG132 treatment did not significantly affect the transcription driven by the pCMV promoter, indicating that MG132 inhibition of VP16 activator-mediated transcription was specific (Fig. 1D). These results demonstrate that the impairment of proteasome function by MG132 not only leads to accumulation of these molecules, but also affects their ability to conduct transcription; and also indicate that proteasome function is required for efficient transcriptional activation by these activators. Since the potency of transcriptional activators is known to correlate with their ubiquitylation status [3], it is easy to surmise that the degradation of ubiquitylated conjugates is linked to transcriptional activation events of this family of synthetic transcriptional activators.

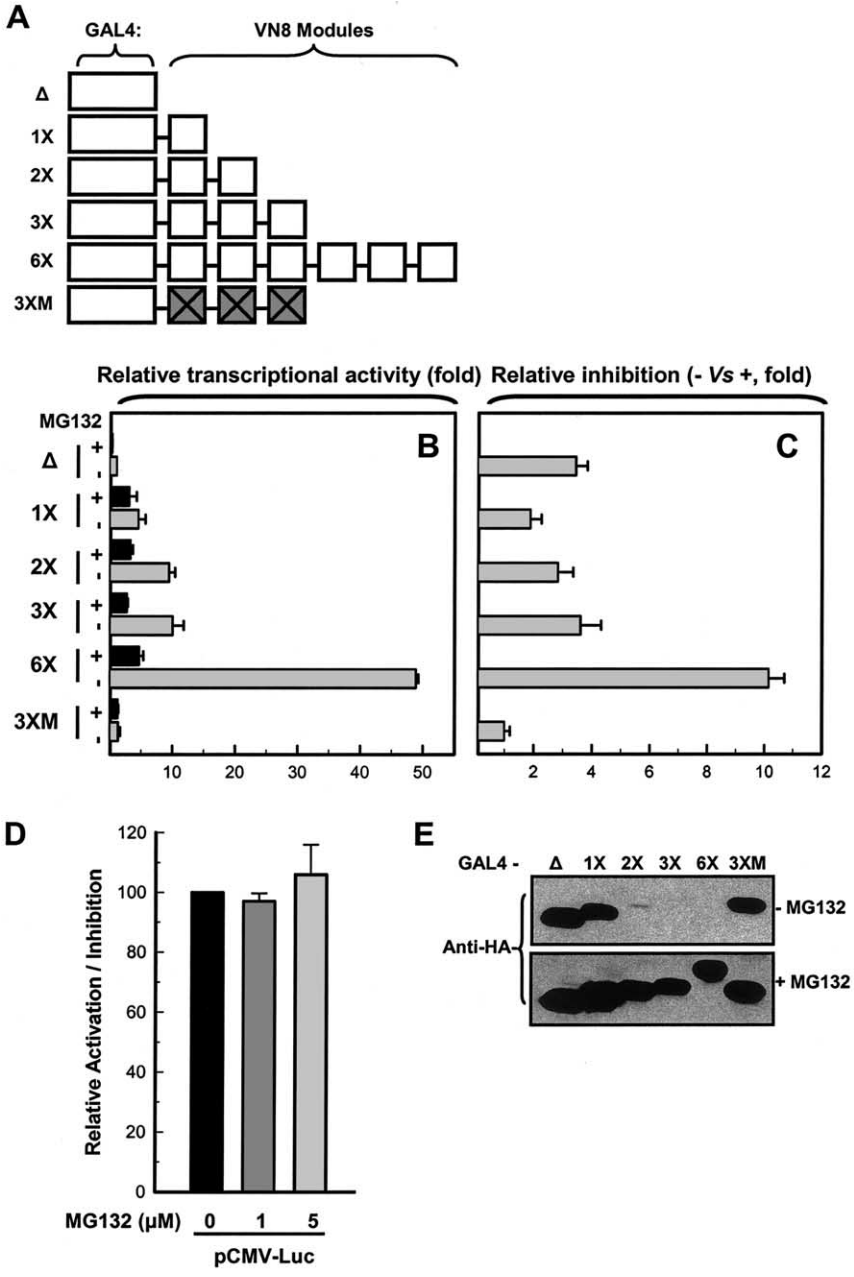

Fig. 1. Proteasome inhibitor MG132 inhibits the degradation of synthetic activators with tandem reiteration of VN8 modules, and reduces their transcriptional activities. A: Schematic presentation of synthetic activators used in this study. Each synthetic activator contains the GAL4 DNA BD fused to 1×, 2×, 3×, or 6× copies of the wild type VN8 sequence or three copies of mutant VN8 sequence (3×M) as previously described. B: The inhibitory effects of MG132 on transactivation mediated by synthetic activators are closely correlated to their potencies of transcriptional activation. 1 μg of pG5-*luc* and 1 μg of expression vector for each synthetic activator were cotransfected into HeLa cells. Twelve hours after transfection, the cells were treated with 5 μM MG132 or its vehicle DMSO for 12 h and the cells collected for luciferase assay. The transient transfection/reporter assay was performed at least three times. The luciferase results are normalized against total protein and relative transcriptional activity is calculated and presented in relation to the basal transcriptional activity of GAL4 DNA BD (Δ). C: The values calculated from normalized luciferase activity in the presence vs. absence of MG132 are shown to present relative inhibition by MG132 for each synthetic activator. D: 1 μg of pCMV-*luc* was transfected into HeLa cells. Twelve hours after transfection, the cells were treated with 1 or 5 μM MG132 or its vehicle DMSO for 12 h and the cells collected for luciferase assay. E: Steady-state levels of synthetic activators in transfected cells. HeLa cells were transiently transfected with expression vector for each synthetic activator and the transfected cells were treated either with 5 μM MG132 or with its vehicle DMSO. Proteins (60 μg) of each sample from parallel experiments were used for Western analysis with anti-HA antibodies.

3.2. *Ubiquitylation is required for efficient transcriptional activation mediated by GAL4-VN8 activators*

The impact of impairment of Ub-proteasome function on transcription mediated by GAL4-VN8 activators was further substantiated in the ts85 cell line (Fig. 2). These cells harbor a temperature-sensitive mutation in UBA which leads to a defect in protein ubiquitylation and accumulation of cellular proteins at the restrictive temperature [8]. Under permissive temperature conditions (32°C), a consistent reverse correlation between transcriptional potency and the steady-state level of

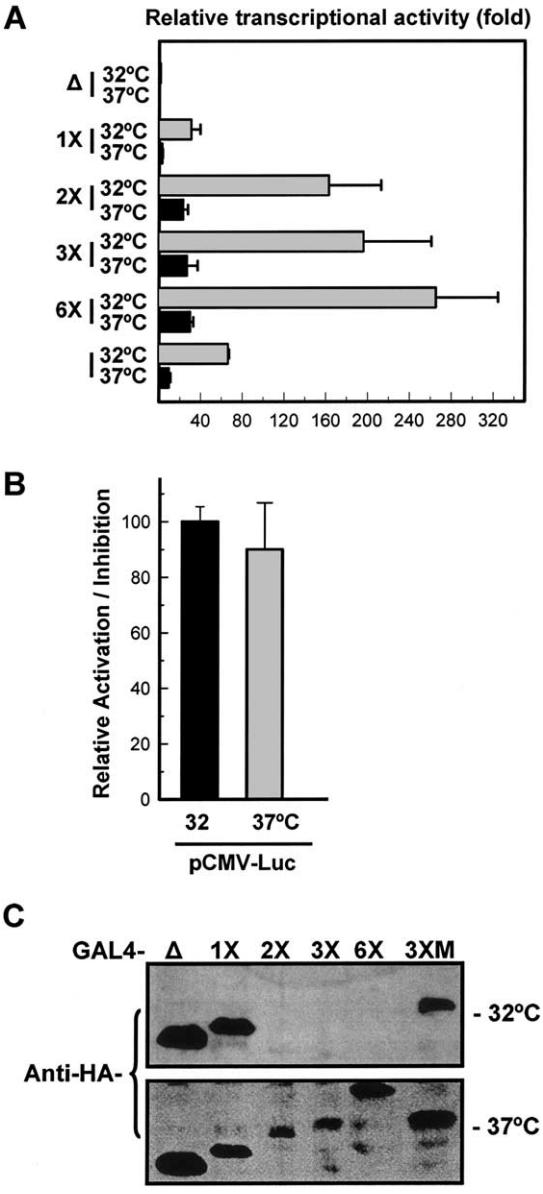

Fig. 2. Disruption of UBA function leads to an increase in steady-state levels of unstable synthetic activators but abrogates their transcriptional function. A: ts85 cells were transiently cotransfected with 10 μg of pG5-*luc* and 10 μg of expression vector for each synthetic activator for 24 h at 32°C. After transfection, cells were divided and maintained at either 32°C or 37°C in fresh medium and harvested for luciferase assay 24 h thereafter. The luciferase assay data were first normalized against total protein and the relative transcriptional activity (fold) calculated and presented in relation to the basal transcriptional activity of GAL4 DNA BD at 32°C. B: ts85 cells were transiently transfected with pCMV-*luc* expression vector for 24 h at 32°C. After transfection, cells were divided and maintained at either 32°C or 37°C in fresh medium and harvested for luciferase assay 24 h thereafter. C: The changes in the steady-state levels of synthetic activators in ts85 cells upon shifting to the restrictive temperature. The ts85 cells were transiently transfected with 10 μg of expression vector for each synthetic activator for 24 h at 32°C and cells were divided and maintained at either 32°C or 37°C as described for A. Proteins (100 μg) of each sample were used for Western blotting.

the activators was also observed in ts85 cells. The steady-state levels of weaker activators, GAL4-Δ, -1× and -3×M, but not of stronger activators, GAL4-2×, -3×, -6×, are clearly detectable by Western analysis of the tagged proteins (Fig. 2A,C, 32°C). GAL4-3×M had a much reduced transcriptional potency compared to GAL4-3×. Its transcription activity and steady-state protein level fall between the values for GAL4-1× and -2×. This unique pattern of transcriptional activation and protein level changed when cells were incubated at the restrictive temperature (37°C). On the one hand, the temperature shift greatly compromised the transcription activity mediated by all these activators, but it had little effect on the transcription mediated through the pCMV promoter (Fig. 2B). For example, incubation at the restrictive temperature (37°C) abolished the transcription by GAL4-1× and -3×M and attenuated the relative transcription activity of GAL4-2×, -3× and -6× from 180-, 200- and 280- to only 40-fold, respectively (Fig. 2). Conversely, incubation at the restrictive temperature (37°C) significantly stabilized these otherwise po-

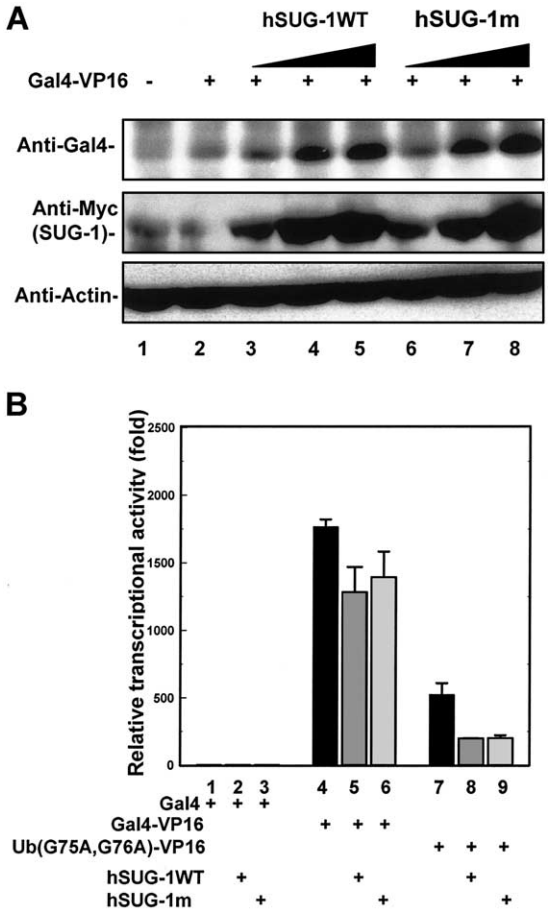

Fig. 3. Overexpression of SUG-1WT and SUG-1m interferes with the degradation and the transactivation by GAL4-VP16 activators. A: 3×10^5 LFS cells were transfected with 0 or 1 μg of GAL4-VP16 expression vector together with increasing amounts (1, 3 or 5 μg) of expression vector for either Myc-tagged SUG-1WT (lanes 3–5) or SUG-1m (lanes 6–8) for 48 h. Equal amounts of proteins from transfected cells were immunoblotted with GAL4, Myc or actin specific antibodies. B: 1×10^5 LFS cells were cotransfected with pG5-*Luc* (0.25 μg) and expression vector for GAL4-VP16 or Ub-(G75A,G76A)-VP16 (0.25 μg) without or with expression vector for either Myc-tagged SUG-1WT or SUG-1m (1.5 μg) for 24 h. Luciferase assay was performed as described in Section 2. Representative results from three independent experiments are shown as mean ± S.D.

tent but unstable activators (Fig. 2C). These results demonstrate that a functional ubiquitylation process is critically important for Ub-mediated proteolysis. Importantly, the results indicate that a functioning ubiquitylation process is also required for efficient transcription mediated by these synthetic transcriptional activators, albeit the requirement is not simply related to the transcriptional potency.

3.3. Overexpression of SUG-1, a subunit of the 19S proteasome complex, leads to accumulation of GAL4-VP16 but attenuates its transcriptional activation

Since proteasome activity is required for degradation of the GAL4-VP16 activator, we next investigated whether SUG-1 could be involved in this process. SUG-1 is one of the components of the 19S regulatory complex of the 26S proteasome [18–20]. It is known to directly bind to TAD of GAL4, viral activator VP16 and thyroid hormone and retinoid-X receptor [21–23]. To investigate whether SUG-1 participates in the degradation of the GAL4-VP16 activator, competition experiments were carried out by transfection of GAL4-VP16 expressing constructs with increasing amounts of an expression vector for either SUG-1WT or SUG-1m, which contains a dominant negative K196M mutation at the AAA ATPase module [24,25]. Overexpressed SUG-1 abrogates the degradation of the GAL4-VP16 activator (Fig. 3A, lanes 2–5). A similar effect was seen with the overexpression of SUG-1m (Fig. 3A, lanes 2, 6–8). The observed effect of SUG-1m is clearly specific because the levels of actin control were unaffected. Despite the elevated GAL4-VP16 levels, overexpression of SUG-1WT or SUG-1m reduces the transcriptional activation to ~70% of the activator control alone (Fig. 3B, lane 4 vs. 5, 6). The results indicate that ectopically overexpressed SUG-1WT and SUG-1m are acting as competitors for an endogenous SUG-1 containing complex, which is involved in both GAL4-VP16 degradation and GAL4-VP16-mediated transcriptional activation.

To examine the role of Ub in GAL4-VP16-mediated transcriptional activation, an Ub containing activator, Ub(G75A, G76A)-VP16, was included in the experiments for transient and reporter assay. Single Ub was directly fused to the N-terminus of GAL4-VP16 activators to generate the corresponding expression constructs. C-terminal glycine residues (glycine 75 and 76) of the Ub moiety in the activator were converted to alanine residues to slow down the removal of Ub by isopeptidases. Such double mutation effectively prevents Ub from being cleaved. The Ub(G75A,G76A)-VP16 activator appears larger in size than parental GAL4-VP16 molecules and the steady-state level of the activator remains comparable to that of parent molecules (data not shown). Surprisingly, the Ub(G75A,G76A)-VP16 activator had lower transcriptional activation potency compared to that of parental GAL4-VP16 molecules (Fig. 3B, lane 4 vs. 7). Seemingly, non-removable Ub exerts a negative structural effect on GAL4-VP16 molecules. Nevertheless, overexpressed SUG-1 and SUG-1m had an enhanced inhibitory effect on the transcriptional activation by Ub(G75A,G76A)-VP16 activators. Overexpression of SUG-1 and SUG-1m caused ~30% and 60% decrease in the transcriptional activation by VP16 and Ub(G75A, G76A)-VP16 (Fig. 3B, lanes 4–6 and 7–9), respectively. The results strongly indicate that the Ub moiety plays a role in recruiting a SUG-1 containing complex for transcriptional activation.

3.4. Ub and Ubl domains of hHR23A retain proteasome-bound general transcription factor (TFIIH)

To demonstrate the physical association of the proteasome with general transcription factors, we applied a unique GST pull-down assay using GST fusion proteins containing either the Ub or the Ubl domain of hHR23A. The Ubl domain has been reported to be a specific ligand for the proteasome through its interaction with the S5a (Rpn10 in yeast) subunit of the 19S proteasome complex [26]. Ub, on the other hand, binds the S2 (Rpn1 in yeast) subunit of the 19S proteasome

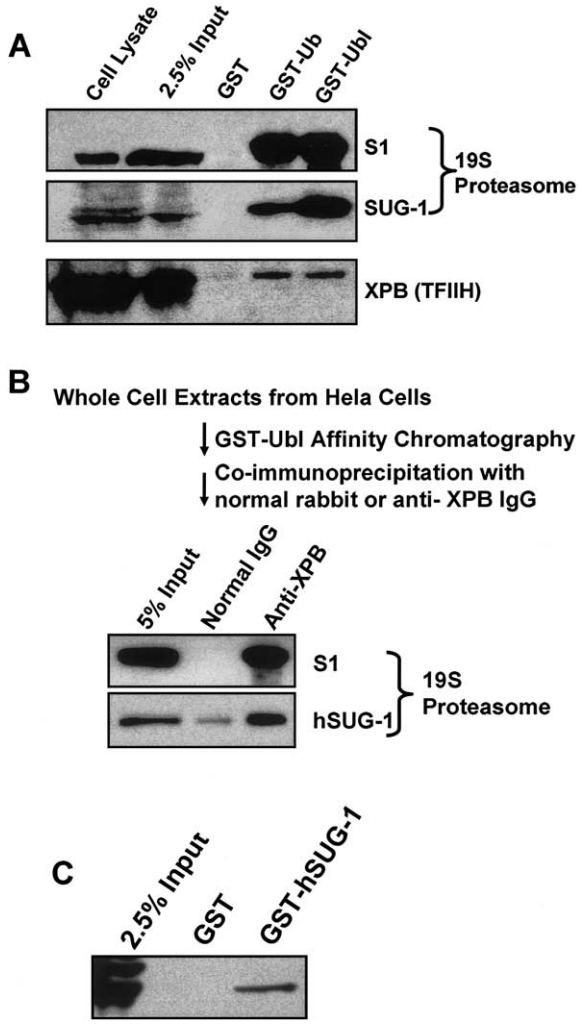

Fig. 4. Physical interactions between proteasome and general transcription factor TFIIH. A: Both Ub and Ubl of hHR23A retain the proteasome and general transcription factor TFIIH. WCE made from HeLa cells were incubated with GST or GST-Ub or GST-Ubl fusion proteins bound to glutathione Sepharose beads. After a thorough washing, the retained proteins were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and immunoblotted with antibodies against 19S proteasome components, S1 and SUG-1 as well as antibody against TFIIH component, XPB. B: Direct association between 19S proteasome and TFIIH. WCE were subjected to GST-Ubl affinity chromatography for Ubl binding proteins. The bound proteins were eluted together with GST-Ubl and co-immunoprecipitated with either normal rabbit IgG or anti-XPB antibody. Immunoprecipitates were detected for the 19S proteasome components S1 and SUG-1. C: SUG-1 interacts with XPB in vitro. HeLa WCE were incubated with GST or GST-SUG-1 fusion proteins bound to glutathione beads. The bound proteins were resolved by SDS–PAGE and immunoblotted with antibody against XPB.

complex [27,28]. As expected, both GST-Ub and -Ubl proteins retained S1 as well as SUG-1 subunits of 19S proteasome complex, whereas neither of these two subunits was detected with the GST protein alone (Fig. 4A). Notably, XPB, a subunit of TFIIH, was also detected in both GST-Ub- and -Ubl-bound proteins. Since Ubl of Rad23 does not bind transcription factors directly [29], these results suggest that the proteasome is associated with TFIIH in vivo. To ensure that TFIIH binding was through interaction with the proteasome and not a result of direct binding to Ubl, WCE from HeLa cells were first subjected to GST-Ubl affinity chromatography and then the GST-Ubl-bound fractions were used for immunoprecipitation by an anti-XPB antibody. As shown in Fig. 4B, both S1 and SUG-1 were found to co-immunoprecipitate with TFIIH/XPB proteins. In addition, the pull-down assay using GST-SUG-1 fusion protein further confirmed the interaction of SUG-1 with TFIIH (Fig. 4C). These findings are not only consistent with the previous observation that SUG-1 interacts with TFIIH [30], but also indicate SUG-1 as a subunit of proteasome in action. As the 20S proteasome complex has not been found in the proteasome APIS (ATPase independent of 20S)/transcription factor complex in yeast [29], it appears that in mammalian cells, the 19S proteasome complex, or at least its component subunit(s), is physically associated with TFIIH in vivo.

4. Discussion

Many eukaryotic transcription factors are known to be degraded through the Ub-proteasome pathway. Increasing evidence suggests that the degradation of transcription activators can be associated with their transcriptional activation function [5,31,32]. The possible association of degradation and transcriptional activation processes has been further strengthened by the observed convergence of transcription and proteolytic signaling elements [3]. Such an association has been demonstrated in estrogen-induced receptor α and retinoic acid-induced receptor γ2 transactivation [33,34]. Here, we have demonstrated that both proteasome and ubiquitylation function are required for efficient transcriptional activation mediated by synthetic VP16 activators in mammalian cells. We have also provided functional and biochemical data to suggest that the 19S proteasome complex, or at least a fragment of the 19S subunit, is recruited for the degradation as well as the transcriptional activation by GAL4-VP16 in vivo. These results provide compelling evidence to support the linkage between the processes of proteolysis and accompanying transactivation.

The requirement of ubiquitylation for VP16 activator function has already been demonstrated in yeast [35]. We have now extended this observation to mammalian cells and explored the function of the proteasome in transcriptional activation. Previous studies have shown that linking a single Ub to activator specifically causes transcriptional activation without signaling protein degradation. Based on this finding and the observation that only the 19S regulatory subunit of the proteasome is required for efficient transcription elongation [6], it is imaginable that both ubiquitylation and proteasome have a non-proteolytic function in transactivation. However, the observations provided in this and other studies [33,34] clearly indicate that the proteasome is required for transcriptional activation. Given the fact that impairment of proteasome function by its inhibitor, MG132, leads to accumulation of both native and Ub-conjugated substrates of the proteasome, these observations suggest that the transcription process is associated with proteolysis. Moreover, these observations argue that proteolysis, a natural consequence of ubiquitylation, must occur as transcription continues. This argument is supported by an opposite case for yeast transcription factor Met4. In that case, ubiquitylation of Met4 is directed by SCF(Met30) Ub ligase, but both Met4 and its Ub conjugates are relatively stable. While ubiquitylated Met4 associates with target promoters, it fails to form a functional transcription complex and negatively regulates Met4-mediated transcription [36]. In essence, Ub plays a dual role in transcriptional activation and degradation.

The involvement of the 19S regulatory subunit of the proteasome in transcription elongation provides a rationale for the requirement of ubiquitylation in transcriptional activation. As mentioned earlier, it has been demonstrated that the 19S regulatory subunit is required for the efficient transcription elongation driven by the GAL4-VP16 activator in vitro and that mutation in yeast SUG-1 inhibits the transcription elongation in vivo [6]. Besides, it has recently been shown that a 19S proteasome complex is recruited to GAL1-10 promoter in vivo [7]. These two lines of evidence suggest that attached Ub may enhance the association between activator and 19S proteasome proteins, although activator alone may be capable of binding 19S proteins as seen for GAL4 AD. In this regard, we have shown that the overexpressed proteasome component, SUG-1, competitively reverses the degradation of GAL4-VP16 but attenuates its transcriptional activation. Moreover, overexpressed SUG-1 and SUG-1m had an enhanced inhibitory effect on transcriptional activation by Ub-attached activators. The inhibitory effect of overexpressed SUG-1 and SUG-1m could be explained due to its competition with endogenous SUG-1 or impairment of normal proteasome function. Therefore, these data strongly suggest that Ub plays an accessory role in associating the activator with 19S proteasomal proteins. Moreover, the demonstrated association of the Ub, proteasome and TFIIH further makes this a very likely operating scenario. At the present juncture, the data cannot help to discern the concrete involvement of either the 19S proteasome complex or the individual 19S regulatory subunits or even the complete 26S proteasome. Despite several attempts, we were unable to detect an association of the components of 20S proteasome with TFIIH through immunoprecipitation with anti-XPB after GST-Ubl affinity chromatography (data not shown). It is possible that such association, if any, is unstable. If degradation of the activator must happen, then it is possible to envision that 26S proteasome would also undergo a dynamic reconstruction. The degradation of the activator would allow rapid reprogramming of the transcription or alternatively allow the transcription elongation to proceed [31,32,37]. When the proteolysis is complete, a part of the 26S proteasome, perhaps the 19S regulatory subunit or the base of 19S regulatory subunit, would be engaged with the elongation step of transcription.

In conclusion, the regulation of viral VP16 TAD function, by ubiquitylation and proteasomal protein degradation, revealed in this study provides an important clue about how transcription and factor degradation are tightly connected processes in mammalian cells. A full understanding of the mechanism and the role played by ubiquitin and proteasome

in transcription will need in-depth studies of the events occurring at an activated promoter and the cognate protein complexes engaged in transcription. To address whether non-proteolytic functions of ubiquitylation and proteasome are involved in transcriptional activation, we are currently investigating whether βTrCP (a homolog of yeast Met30) is a component of SCF E3 ligase for GAL4-VP16 and whether βTrCP is recruited to the promoter region for its transactivation.

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