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Proteasome-mediated degradation of transcriptional activators correlates with activation domain potency *in vivo*

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We show that the intracellular concentration of transcriptional activator proteins is regulated by the proteasome-mediated protein degradation pathway. The rate of degradation of activators by proteasomes correlates with activation domain potency *in vivo*. Mutations either in the activation domain residues involved in target protein interaction or in the DNA-binding domain residues essential for DNA binding abolish the transcriptional activation function *in vivo* and render the activator resistant to degradation by proteasomes. Finally, using a rapamycin-regulated gene expression system, we show that recruiting activation domains to DNA-bound receptor proteins greatly enhanced the rate of degradation of reconstituted activators. These observations suggest that in mammalian cells efficient recruitment of activator–target protein complexes to the promoter means that they are subjected to rapid degradation by proteasomes. We propose that proteasome-mediated control of the intracellular levels of transcriptional activators could play an important role in the regulation of gene expression.

Keywords: activation domain potency/degradation/proteasome/transcription/transcriptional activator

Introduction

Transcriptional initiation of eukaryotic genes by RNA polymerase II requires the assembly of several dozen proteins on the promoter region (Choy and Green, 1993; Tjian and Maniatis, 1994; Struhl, 1996). These proteins are classified as transcriptional activators, chromatin-modifying proteins, general transcription factors (GTFs), transcriptional coactivators and the components of the RNA polymerase II holoenzyme complex (Ptashne, 1988; Tjian and Maniatis, 1994; Orphanides *et al*., 1996; Ptashne and Gann, 1996). Within this diverse group of proteins, only the transcriptional activators exhibit a high degree of affinity and binding specificity for DNA sequences. Several components of the transcription machinery have been shown to interact with transcriptional activator proteins *in vitro*. Recent 'activator bypass' experiments show that recruiting the subunits in TFIID or RNA polymerase II holoenzyme complex directly to the promoter itself is sufficient to induce the transcriptional activation of eukaryotic genes (Jiang and Stillman, 1992; Barberis *et al*., 1995; Chaterjee and Struhl, 1995; Klages and Strubin, 1995; Xiao *et al*., 1995; Farrell *et al*., 1996; Gaudreau *et al*., 1998; Keaveney and Struhl, 1998). Together, these findings strongly support the idea that a majority of the proteins required to initiate the transcription of a eukaryotic gene are recruited to the promoter through direct or indirect contact with transcriptional activator proteins (Ptashne and Gann, 1996; Struhl, 1996).

Transcriptional activators, in general, are composed of two highly modular functional domains: an activation domain and a DNA-binding domain (Hope and Struhl, 1986; Ptashne, 1988; Ptashne and Gann, 1990). The DNA-binding domain tethers the activation domain to DNA by binding to a specific nucleotide sequence in the promoter region of a target gene. Numerous studies have established that activation domains interact with one or more subunits of the TFIID complex, various SWI/SNF and SAGA chromatin remodeling complexes, RNA polymerase II holoenzyme, etc. (Peterson and Tamkun, 1995; Farrell *et al*., 1996; Wilson *et al*., 1996; Ptashne and Gann, 1997; Keaveney and Struhl, 1998; Koh *et al*., 1998). Tethering activation domains to a specific promoter is necessary and sufficient for the recruitment of components of the transcription machinery and, subsequently, transcriptional initiation (Ptashne and Gann, 1997; Keaveney and Struhl, 1998; Koh *et al*., 1998). The potency of an activator is thought to depend at least in part on the affinity of the activation domain for one or more components of the transcription machinery (Wu *et al*., 1996).

A large number of transcriptional activators have been cloned and characterized in recent years. However, only a small number of these activator proteins have been shown to function as highly potent inducers of transcription *in vivo*. Notable among these transcriptional activators are the herpes simplex virus protein VP16, the p65 subunit of the human transcription factor NF-κB and the human heat shock factor HSF-1 (Cress and Triezenberg, 1990; Ballard *et al*., 1992; Blair *et al*., 1994; Schmitz *et al*; 1994; Morimoto, 1998). Chimeric transcriptional activators containing either VP16 or p65 activation domains are generally expressed at very low levels in eukaryotic cells (Berger *et al*., 1990, 1992; Blair *et al*., 1994; Shockett *et al*., 1995; Baron *et al*., 1997; E.Molinari and S.Natesan, unpublished data). It is generally thought that the cytotoxicity of potent chimeric transcriptional activator proteins limits their expression in eukaryotic cells. It has been hypothesized that the probable reason for the cytotoxicity of potent transcriptional activators is their ability to titrate essential GTFs that are present in limiting amounts in eukaryotic cells (Gill and Ptashne, 1988; Berger *et al*., 1990, 1992; Natesan *et al*., 1997). However, despite this long-standing view, direct experimental evidence linking the activator's ability to titrate GTFs to its low intracellular levels is not available.

Here we show that chimeric transcriptional activators carrying potent activation domains are degraded rapidly in mammalian cells by the proteasome-dependent protein degradation pathway. Further analysis revealed that the rate of degradation of potent transcriptional activator proteins correlates with the potency of their activation domains. We also show that an activator's ability to interact with its target proteins is both necessary and sufficient for its degradation. Finally, we show that recruiting activation domains to DNA-bound receptor proteins enhances the rate of degradation of both the activation and the DNA-binding domain proteins. The data shown here provide strong evidence for the presence of a proteasome-dependent protein degradation machinery whose function is to recognize and destroy stable, DNA-bound protein complexes containing transcriptional activators and perhaps their associated target proteins.

Results

Inverse correlation between potency and intracellular levels of transcriptional activators

A major goal in our laboratory is to generate transcriptional activators capable of strongly inducing the transcription of genes embedded in chromatin. To achieve this goal, we generated numerous chimeric transcriptional activators and analyzed their ability to induce the transcription of a stably integrated reporter gene in mammalian cells. This cell line, designated HT1080B, carries a single copy of the *SEAP* reporter gene driven by a promoter containing five GAL4-binding sites flanking the minimal interleukin 2 promoter in HT1080 human fibrosarcoma cells (Natesan *et al*., 1997).

We expressed the chimeric activator proteins shown in Figure 1 and measured both their transcriptional activity and intracellular concentration (Figure 1). All activators used in this experiment contained the DNA-binding domain derived from the yeast transcription factor GAL4 (Marmorstein *et al*., 1992). The activators shown in Figure 1 were divided into three groups based on their type of activation domains. The activators in the first group (see left panel) carry activation domains derived from various transcriptional activators. These include acidic activation domains capable of stimulating transcription from distal enhancer elements such as p65 and VP16 activation domains; activation domains capable of inducing transcription only when bound to promoter elements proximal to the TATA-box such as those from p53, serum response factor (SRF) or glutamine-rich activators (SP1, OCT-1); and activation domains known to induce transcription when tethered to both proximal promoter elements and distal enhancer regions such as the proline-rich activation domain of CAAT box binding transcription factor (CTF) (Courey and Tjian, 1988; Cress and Triezenberg, 1990; Ballard *et al*., 1992; Seipel *et al*., 1992; Lin *et al*., 1994; Tanaka *et al*., 1994; Das *et al*., 1995; Joliot *et al*., 1995; Blau *et al*., 1996; Uesugi *et al*., 1997). The second group of activators carry two or more copies of an 8-amino-acid peptide, DFDLDMLG, derived from the VP16 activation domain (middle panel; Tanaka, 1996). Activators in the third group carry either a p65 activation domain of varying length or a p65 activation domain fused with a synergizing activation domain, such as V8×8 (right panel). In each group, the intracellular concentration of activators that strongly stimulated expression of *SEAP* was much lower than the concentration of activators that induced the reporter gene only slightly above background level (compare top and bottom panels in Figure 1 showing the transcriptional activity and the intracellular concentration of the activator, respectively). These observations reveal the presence of an inverse correlation between the intracellular levels of chimeric transcriptional activator proteins and their activation domain potency *in vivo*.

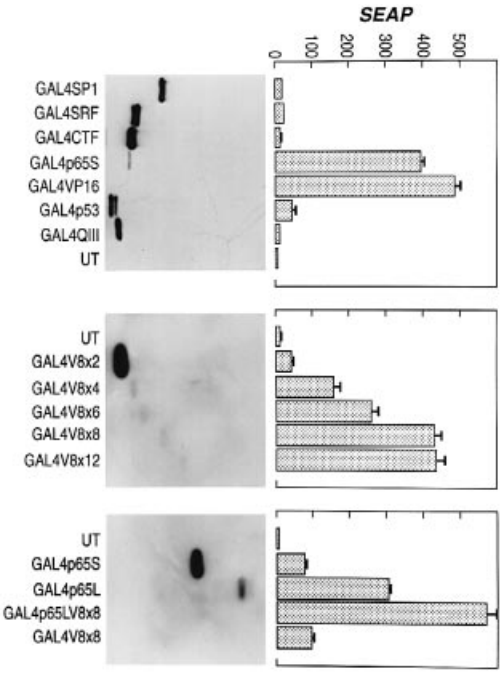

Fig. 1. Strict inverse correlation between the potency and intracellular levels of chimeric transcriptional activator proteins. HT1080B cells were transfected with expression plasmids encoding the indicated activator protein and, ~24 h post-transfection, the SEAP activity in the medium was measured. For the experiment shown in the top panel, activators representing acidic activation domains (VP16, p65), glutamine-rich activation domains (SP1, OCT-1 QIII), a proline-rich activation domain (CTF) and activation domains that interact with TFIIF (SRF) were used. For the experiment shown in the middle panel, activators carrying 2–12 copies of the V8 domain (DFDLDMLG) were used. For the experiment shown in the bottom panel, activators carrying varying lengths of the p65 activation domain of or the p65 activation domain fused with V8×8 were used. Mean values of SEAP activity secreted into the medium are shown (± SD). In each case, extracts from transfected and control cells were subjected to Western blot analysis using an appropriate antibody. The Western blot in the first panel was carried out using GAL4 antibody. The other two blots were probed with HA antibody (Babco).

Potent transcriptional activator proteins are degraded rapidly

To identify the basis for this correlation, we first examined whether the mRNA levels of transcriptional activators correlate with their intracellular concentration. For this analysis, we introduced expression plasmids encoding GAL4, GAL4V8×2, GAL4V8×4 and GAL4V8×8 activators into cells and analyzed their steady-state mRNA levels by RNase protection assays. These activators carry two or more copies of an 8-amino-acid motif, DFDLDMLG, derived from the VP16 activation domain

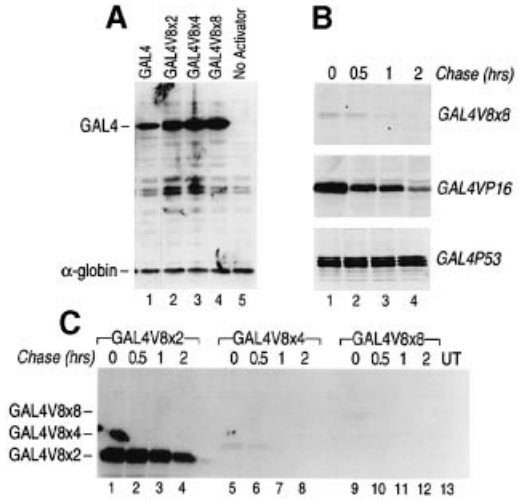

Fig. 2. Rate of degradation of chimeric activator proteins correlates with the strength of the activation domain. (**A**) Total cellular RNA prepared from cells transfected with the indicated plasmids was subjected to RNase protection analysis. (**B**) Pulse–chase analysis of GAL4V8×8, GAL4VP16 and GAL4p53 activator proteins in HT1080B cells. Expression plasmids for these activators were transfected into HT1080B cells. Approximately 18 h after transfection, methionine-free fresh medium containing [^{35}S]methionine–cysteine mix was added to cells. After a 1 h pulse, the medium was replaced with normal medium containing 1 mM methionine. Cells were harvested at the indicated times and the lysates prepared were subjected to immunoprecipitation using HA antibody. (**C**) Pulse–chase analysis of indicated proteins was carried out as described in (B).

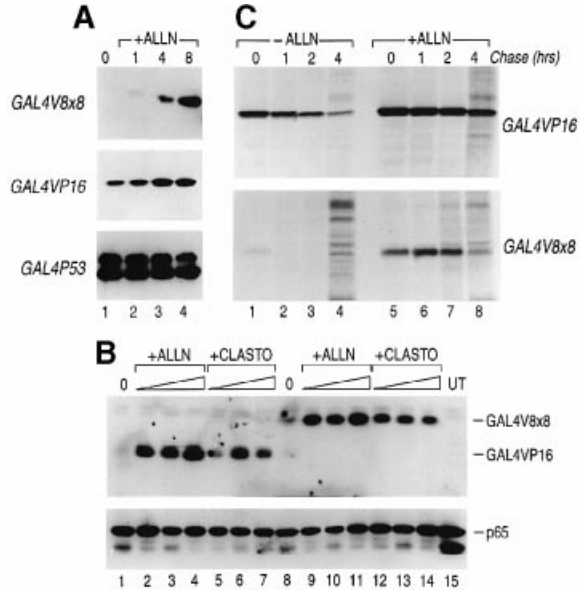

Fig. 3. Potent transcriptional activators are degraded rapidly by proteasomes. (**A**) HT1080B cells expressing GAL4V8×8, GAL4VP16 or GAL4P53 activator proteins were treated with ALLN for the indicated periods of time. Total cell lysates from transfected cells were subjected to Western blot analysis using HA antibody. (**B**) HT1080B cells expressing GAL4VP16 or GAL4V8×8 were treated with increasing concentrations of two proteasome inhibitors, ALLN and clasto-lactacystin, for 6 h. Cell lysates were subjected to Western blot analysis using HA antibody. The membrane was reprobed with p65 antibody to confirm that a roughly equal amount of protein is loaded in each lane. (**C**) Expression plasmids encoding GAL4VP16 and GAL4V8×8 activators were introduced into HT1080B cells by transient transfection. Approximately 18 h later, pulse–chase analysis was carried out as described above. Immunoprecipitation of the HA-tagged activators from cell lysates was carried out using HA antibody as described above.

(Tanaka, 1995). We chose this set of activators for two reasons. First, the activation domains in these activators are composed of qualitatively similar units and their potency *in vivo* correlates with the number of V8 units. Secondly, these activators exhibit an inverse correlation between activation domain potency *in vivo* and intracellular concentration (see Figure 1, middle panel). A representative RNase protection experiment shown in Figure 2A reveals that despite the variations in their potency and intracellular levels, the mRNAs were present at comparable levels. Thus, the intracellular protein concentration of transcriptional activators does not correlate with their mRNA levels. This finding led us to conclude that the regulation of the intracellular concentration of potent activator proteins must occur at either the translational or the post-translational level.

To investigate whether rapid turnover of potent chimeric transcriptional activator proteins could explain their low intracellular levels, we measured the half-life of GAL4VP16, GAL4V8×8 and GAL4p53 activators by pulse–chase analysis. Both GAL4VP16 and GAL4V8×8 function as potent inducers of transcription of the stably integrated reporter gene, whereas GAL4p53 induced the reporter gene only modestly above the background level (see Figure 1). In the pulse–chase experiment, we observed that both GAL4V8×8 and GAL4VP16 were degraded very rapidly, with approximate half-lives of 15 and 60 min, respectively (Figure 2B). In contrast, the weak activator, GAL4p53, remained stable for >4 h (Figure 2B). These observations suggest that highly potent activators such as GAL4VP16 and GAL4V8×8 are degraded rapidly in mammalian cells and that the rate of degradation of activator proteins generally correlates with activation domain potency *in vivo*.

To seek further evidence in support of this conclusion, we measured the half-life of another set of activator proteins containing GAL4V8×2, GAL4V8×4 and GAL4V8×8. The data from a representative pulse–chase experiment are shown in Figure 2C. The data illustrate that the rate of degradation of activators correlated with the number of V8 domains in the activator, which in turn correlates with potency *in vivo*. These observations further support the idea that the rate of degradation of an activator correlates with activation domain potency *in vivo*.

Potent transcriptional activators are degraded rapidly by proteasomes

The intracellular expression level of many eukaryotic transcription factors has been shown to be regulated through rapid degradation by the proteasome-mediated protein degradation pathway (Mak *et al*., 1996; Mathew *et al*., 1998; Mitsui and Sharp, 1999; Nawaz *et al*., 1999). To determine whether the degradation of potent chimeric activators is mediated by proteasomes, we measured the steady-state levels of GAL4VP16, GAL4V8×8 and GAL4p53 proteins in the presence of the proteasome inhibitor peptide aldehyde, *N*-acetyl-leucinyl-leucinyl-norleucinal-H (ALLN). We observed that treating cells that express GAL4VP16 and GAL4V8×8 activators with the proteasome inhibitor ALLN led to a significant increase in the intracellular concentration of these activator proteins

(Figure 3A). This observation suggests that the proteasome-dependent degradation pathway could play a role in the regulation of the intracellular levels of potent transcriptional activators. In addition to ALLN, we observed that another proteasome inhibitor, lactacystin (Fenteany *et al*., 1995), could also block the degradation of GAL4VP16 and GAL4V8×8, suggesting that multiple proteases in the proteasome complex participate in the degradation of potent transcriptional activators (Figure 3B). To assess the effect of proteasome inhibitors on the rate of degradation of activators, we measured the half-life of GAL4VP16 and GAL4V8×8 activators in the presence of ALLN. Data shown in Figure 3C reveal that the half-life of these activators increased significantly in the presence of the proteasome inhibitor. From these observations, we conclude that the intracellular levels of potent transcriptional activator proteins are recognized and degraded rapidly by the proteasome-dependent protein degradation pathway.

Point mutations that abolish the function of the activation domain protect the activator from degradation

Taken together, the data shown above establish a direct link between the potency of activator proteins *in vivo* and their rate of degradation by proteasomes. This raises the possibility that mutations in the activation domain that abolish an activator's ability to induce transcription may provide immunity against proteasome-mediated degradation. To test this possibility, we generated a mutant V8 peptide, V8(F-A), in which the critical phenylalanine in the V8 peptide was replaced with an alanine residue (Figure 4A). This substitution has been shown previously to abolish the transcriptional activity of V8 peptide (Tanaka, 1996). We expressed GAL4 fusion proteins containing four or eight copies of either the mutant or wild-type V8 peptide, GAL4V8×4(F-A), GAL4V8×8 (F-A), GAL4V8×4 and GAL4V8×8, respectively, and analyzed their transcriptional activity *in vivo*. As expected, we observed that both wild-type activators, GAL4V8×4 and GAL4V8×8, induced the expression of the reporter gene very strongly, whereas GAL4V8×4(F-A) and GAL4V8×8(F-A) induced the reporter gene only marginally above background (Figure 4D). This observation confirms our prediction that the phenylalanine residue in the V8 peptide is essential for its transcriptional activation function *in vivo*.

Next we asked whether the loss of transcriptional activation function could protect the mutant activator protein from degradation by proteasomes. We measured the steady-state levels of the mutant and wild-type GAL4V8×4 or GAL4V8×8 fusion proteins in HT1080B cells. As observed in previous experiments, wild-type activators, GAL4V8×4 and GAL4V8×8, were present at undetectable levels. In contrast, the mutant proteins GAL4V8×4(F-A) and GAL4V8×8(F-A) were present at very high levels in the cell (Figure 4B). Pulse–chase analysis of the half-life of GAL4V8×8(F-A) and GAL4V8×8 fusion proteins confirmed that the mutant proteins were resistant to degradation by proteasomes and thus remained present for prolonged periods of time (Figure 4C). Together, these observations demonstrate that mutations that abolish the activator's ability to induce transcription offer immunity against proteasome-mediated degradation.

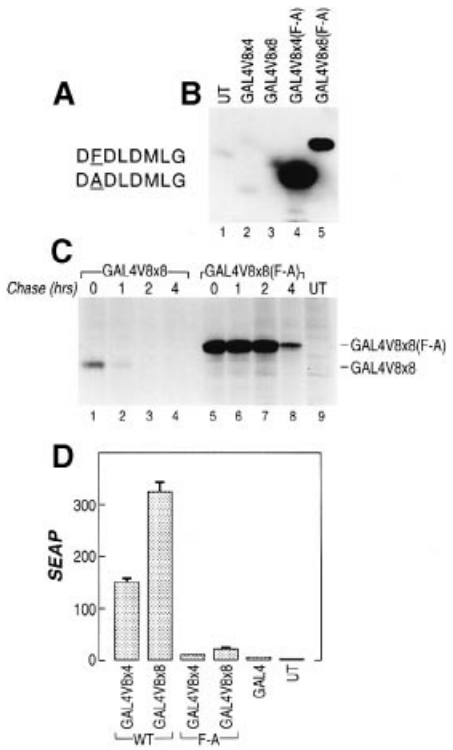

Fig. 4. A mutation in the activation domain which abolishes the activator's ability to induce transcription provides complete immunity from degradation by proteasomes. (**A**) Sequence of the wild-type and mutant V8 activation domain peptide. (**B**) Expression plasmids encoding the indicated wild-type and mutant activator proteins were transiently transfected into HT1080B cells. Approximately 18 h after transfection, cells were harvested and the lysates prepared from these cells were used in Western blot analysis. The membranes were probed with HA antibody. (**C**) Plasmids encoding GAL4V8×8 and GAL4V8×8(F-A) proteins were introduced into HT1080B cells by transient transfection. Approximately 18 h after transfection, pulse–chase analysis was carried out as described above and HA-tagged chimeric activators were immunoprecipitated using HA antibody. (**D**) The indicated expression plasmids encoding wild-type and mutant activator proteins were introduced into HT1080B cells by transient transfection. Approximately 18 h post-transfection, the SEAP activity in the medium was measured. In each case, the mean SEAP value is shown (± SD).

Recruiting activation domains to the promoter enhances the degradation of activators

Next we investigated whether abolishing the DNA-binding activity of the activator can also offer protection from degradation by proteasomes. For this experiment, we generated two mutant GAL4 DNA-binding domains: GAL4M2 and GAL4M3. We expressed the wild-type and mutant GAL4 DNA-binding domains either alone or as fusion proteins carrying the V8×8 activation domain in HT1080 cells. Nuclear extracts prepared from transfected cells were used to analyze the DNA-binding activity of the GAL4 fusion proteins. This analysis showed that the DNA-binding activity of fusion proteins carrying either GAL4M2 or GAL4M3 mutations in the DNA-binding domain was severely impaired compared with the wild-type fusion protein (Figure 5A). In this experiment, the amount of GAL4V8×8 fusion protein present in the nuclear extract is so low that its binding to GAL4 probes

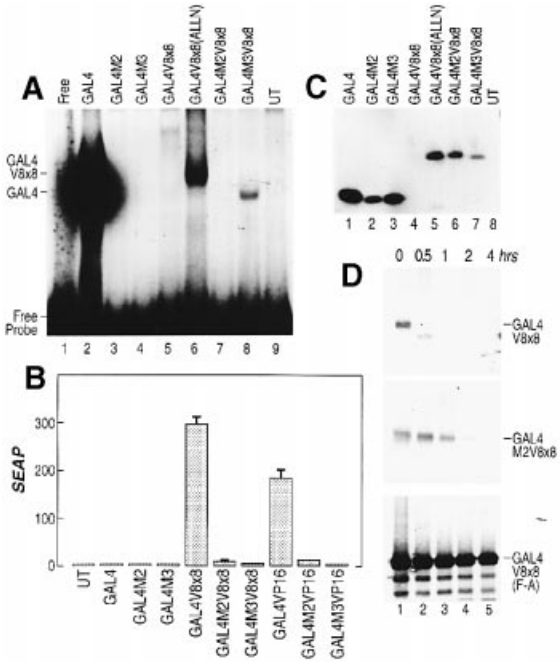

Fig. 5. Mutations in the DNA-binding domain protect the activator from degradation by proteasomes. (**A**) HT1080B cells were transiently transfected with the indicated expression plasmids and, 18 h post-transfection, cells were harvested and nuclear extracts were prepared. The ability of the chimeric proteins to bind to GAL4 probe was tested by gel mobility shift assay. The positions of GAL4 and GAL4V8×8–DNA complex and free GAL4 probes are shown. (**B**) The indicated expression plasmids were transfected into HT1080B cells by transient transfection and, 18 h later, the SEAP activity secreted into the medium was measured. The mean SEAP values are shown in each case (± SD). (**C**) Western blot analysis of lysates from cells expressing the indicated chimeric activator proteins. The membranes were probed with HA antibody. The plasmids encoding the chimeric activators were introduced into HT1080B cells by transient transfection. (**D**) Pulse–chase analysis of the indicated chimeric activator proteins. Plasmids encoding the indicated activator proteins were transiently transfected into HT1080B cells, and pulse–chase and subsequent immunoprecipitation with HA antibody experiments were carried out as described above.

is undetectable (Figure 5A). However, nuclear extracts from cells expressing GAL4V8×8 treated with the proteasome inhibitor ALLN showed high levels of DNA-binding activity (Figure 5, compare lanes 5 and 6; see Figure 4 for the effect of ALLN on the intracellular levels of GAL4V8×8 protein). Consistent with their inability to bind to GAL4-binding sites *in vitro*, GAL4M2V8×8 and GAL4M3V8×8 activators also failed to induce the transcription of the stably integrated reporter gene (Figure 5B).

To examine the effect of the DNA-binding domain mutations on the stability of the activators, we analyzed the steady-state levels of GAL4V8×8, GAL4M2V8×8 and GAL4M3V8×8 fusion proteins. The data in Figure 5C show that the fusion proteins that are unable to bind to GAL4-binding sites, GAL4M2-V8×8 and GAL4M3-V8×8, were present at much higher levels than the wild-type activator, GAL4V8×8 (compare lanes 6 and 7 with lane 4). The level of mutant activator proteins appeared to be comparable with the amount of GAL4V8×8 when their degradation is inhibited by the proteasome inhibitor ALLN. These observations suggest that mutations in the DNA-binding domain, similarly to the mutations in the activation domain, abolish an activator's ability to induce transcription and provide protection from proteasome-mediated degradation.

To compare the effects of the DNA-binding domain and activation domain mutations on the rate of turnover of the activator proteins, we measured the half-life of GAL4V8×8, GAL4M2V8×8 and GAL4V8×8(F-A) fusion proteins. We observed that GAL4M2V8×8, which binds to GAL4-binding sites very poorly, was degraded rapidly, albeit at a slower rate than the wild-type activator GAL4V8×8 (Figure 5D). In contrast, the activator with mutations in the activation domain was much more resistant to degradation than the wild-type activator or the activator with a mutant DNA-binding domain [compare GAL4M2V8×8 and GAL4V8×8(F-A) in Figure 5]. This observation suggests two conclusions. First, proteasome-mediated degradation of activators is dependent primarily on the activators' ability to interact with their target proteins. Secondly, DNA-bound activator–target protein complexes are recognized and degraded by proteasomes more efficiently than activator–target complexes not bound to DNA, perhaps because these complexes are highly unstable.

Recruitment of activation domains to DNA enhances proteasome-mediated degradation

Collectively, the data shown above suggest that potent activation domain fusion proteins must be tethered to their binding sites in the genome in order to be recognized efficiently by the proteasome-mediated protein degradation pathway. To assess this possibility directly, we utilized the rapamycin-regulated gene expression system (Rivera *et al*., 1996). In the system used here, the GAL4 DNA-binding domain and the p65 activation domain were fused with the ligand-binding domains FKBP12 and FRB, respectively. The small molecule drug rapamycin binds with high affinity to both FKBP12 and FRB domains and therefore can recruit the activation domain fusion protein to the DNA-bound GAL4 receptor protein.

Figure 6A shows the effects of recruiting the activation domains to a DNA-bound receptor protein on the intracellular concentration of reconstituted activators. In this experiment, we expressed the DNA-binding domain and activation domain fusion proteins, GF4 and RS, respectively, in HT1080B cells and measured the stability of these fusion proteins in the presence or absence of rapamycin. This analysis showed that in the absence of rapamycin in the medium, the DNA-binding receptor protein (GF4) and the activation domain fusion protein (RS) were present at readily detectable levels when expressed either alone or together (Figure 6A). The presence of 10 nM rapamycin in the medium had no effect on the intracellular levels of GF4, RS and R (R = FRB domain alone) proteins when they were expressed separately in cells. In contrast, when GF4 and RS fusion proteins were co-expressed, the presence of rapamycin in the medium caused a substantial decline in the intracellular levels of both GF4 and RS fusion proteins (Figure 6A, lanes 3 and 4). In contrast, adding rapamycin to the medium had no effect on the intracellular levels of co-expressed fusion proteins GF4 and R. This observation implies that the recruitment of a potent activation domain to DNA leads to the increased degradation of the reconstituted

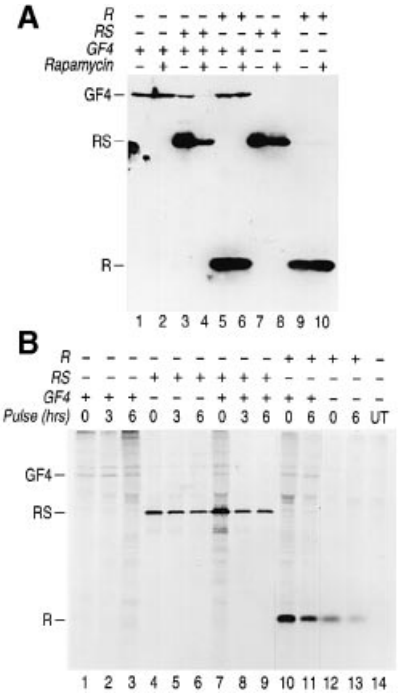

Fig. 6. Tethering the activation domain to the DNA-binding domain triggers the degradation of the reconstituted activator. (**A**) HT1080B cells were transiently transfected with expression plasmids encoding fusion proteins GF4, RS and R either separately or in combination with others. Rapamycin to a final concentration of 10 nM or the carrier solution (ethanol) was added to the medium at the time of transfection. After 18 h, cells were harvested and the extracts prepared were subjected to immunoblot analysis using 12CA5 antibody. (**B**) The indicated expression plasmids were transiently transfected into HT1080B cells and, 24 h later, the cells were refed with fresh serum-free, methionine-free medium for 1 h. Later, cells were incubated in methionine-free medium containing 100 μCi of [^{35}S]methionine and [^{35}S]cysteine mix for 1 h. Cells were refed with fresh medium containing 1 mM cold methionine and kept in this medium at 37°C for the indicated periods of time. Lysates prepared from these cells were used to immunoprecipitate the HA-tagged recombinant proteins.

activator proteins and perhaps other factors associated with them. Pulse–chase analysis of GF4 and RS fusion proteins in the presence or absence of rapamycin also confirmed that these fusion proteins, when expressed together, undergo rapid degradation in the presence of rapamycin in the medium (Figure 6B). Taken together, these observations indicate that activation domain fusion proteins undergo only mild degradation when they are not tethered to their binding sites, whereas their recruitment to the binding sites in the genome greatly enhances the degradation of the non-covalently linked reconstituted activator protein (Figure 6C). Furthermore, these observations also suggest the possibility that other factors associated with the activation domains may also be targeted for degradation by proteasomes.

Discussion

We have demonstrated that intracellular levels of transcriptional activator proteins are regulated by the proteasome-mediated protein degradation pathway. Proteasome-mediated degradation of activator proteins is dependent primarily on activation domain function *in vivo*, whereas abolishing the DNA-binding function only provides partial immunity from degradation. Finally, recruiting activation domains to the DNA-bound receptor protein enhances the degradation of both the activation domain and DNA-binding domain fusion proteins. These observations suggest that DNA-bound, stable activator–target protein complexes are degraded very efficiently by proteasomes. We propose that degradation of stable activator–target protein complexes formed on the promoter and other non-specific regions in the genome may have a significant impact on the program of gene expression in eukaryotic cells.

Our observations show that the integrity of the activation domain is essential for the proteasome-mediated degradation of activator molecules. For example, a phenylalanine to alanine mutation in the V8 activation domain not only abolishes the ability of GAL4V8×8 activator to induce transcription, but also protects it from degradation. The potency of activation domains *in vivo* has been shown to correlate generally with affinity for the components of the general transcription machinery *in vitro* (Blair *et al.*, 1994; Melcher and Johnston, 1995; Wu *et al*., 1996; Ptashne and Gann, 1997). For example, mutations in the VP16 activation domain which reduce its affinity for TFIID *in vitro* also reduce its ability to induce transcription *in vivo* (Ingles *et al*., 1991; Triezenberg, 1995). Similarly, mutations in the p65 activation domain that reduce potency *in vivo* also appear to interact poorly with GTFs *in vitro* (Blair *et al.*, 1994; S.Natesan, unpublished data). Consistent with this, we have found that replacing the phenylalanine with an alanine in the V8 activation domain results in a significant reduction in its ability to interact with at least one potential target, TFIIB, *in vitro* (data not shown). Taken together, it is reasonable to conclude that the affinity of the transcriptional activator proteins for the components of the transcription machinery *in vivo* may determine their rate of degradation by proteasomes.

We have shown that DNA-bound activators are recognized more efficiently by proteasomes than are unbound activator proteins. The 'triangle' model proposed by Struhl (1996) predicts that activator binding to DNA could facilitate the recruitment of GTFs and subsequently the formation of stable activator–GTF complex. If this is the case, one reason for the rapid degradation of DNA-bound activators could be that proteasomes can recognize efficiently only the stable activator–target complexes on the DNA. Alternatively, degradation of potent activator proteins is a consequence of downstream events that require DNA binding, such as the initiation of transcription by RNA polymerase II. This does not appear to be the case because treating cells with α-amanitin, an inhibitor of RNA polymerase II, failed to protect the activators from degradation by proteasomes. Thus, it is possible that DNA binding facilitates the formation of stable activator–target complexes, which are recognized and degraded efficiently by proteasomes.

Role of proteasomes in gene regulation

The proteasome-dependent protein degradation pathway has been shown to modulate the intracellular levels of several regulatory proteins implicated in the control of key cellular functions including cell cycle progression, signal transduction, differentiation, programed cell death and regulation of transcription (Scheffner *et al*., 1993;

Ciechanovar, 1994; Alkalay *et al*., 1995; Jentsch and Schlenker, 1995; Murray, 1995; Pagano *et al*., 1995; Chen *et al*., 1996; Kim and Maniatis, 1996; Pariat *et al*., 1997; Baumeister *et al*., 1998; Hirsch *et al*., 1998; Mathew *et al*., 1998; Mitsui and Sharp, 1999; Nawaz *et al*., 1999). The finding that proteasomes modulate the intracellular levels of potent transcriptional activators further strengthens the view that proteasomes play a crucial role in the regulation of intracellular levels of a wide range of regulatory proteins in the cell. In eukaryotic cells, numerous activators may compete simultaneously for the same target proteins in the nucleus. The data shown here suggest that proteasomes recognize only those activators that are associated with their target proteins. This raises the question of how proteasomes specifically recognize certain activator–target protein complexes but not others. Because only DNA-bound activators with strong activation domains are degraded efficiently by proteasomes, it is possible that the recognition of activator–target protein complexes by proteasomes is based solely on the stability of the complex.

Rapid degradation of stable activator–target protein complexes bound to specific and non-specific sites in the genome may be necessary to maintain the integrity of the global transcription process. For example, the human genome may contain numerous binding sites for activator proteins outside of gene-specific promoter regions. Recruitment of activators and their associated proteins to these sites, if unchecked, could trap GTFs and other components of the transcription machinery that are present in limiting amounts in the cell, ultimately leading to cell death. It is possible that the proteasome-mediated protein degradation pathway plays a key role in alleviating this problem.

Stable activator–GTF complexes are more likely to form on promoters that contain binding sites for potent activators. Many inducible transcription factors function as highly potent inducers of transcription *in vivo* and, therefore, their binding to target promoters could facilitate the formation of stable pre-initiation complexes. If these stable activator–target protein complexes are indeed recognized and degraded rapidly by proteasomes, at least on these promoters frequent assembly of a pre-initiation complex would be necessary to direct a high level of transcription of the target gene.

By modulating the intracellular levels of transcriptional activators, the proteasome-mediated protein degradation pathway may also play an important role in the regulation of extracellular signal-induced gene expression. Many natural transcriptional activators such as NF-κB, STATs and heat shock factor proteins (HSFs) remain in a latent state in the cytoplasm and translocate to the nucleus to induce their target genes in response to extracellular signals (Baldwin, 1996; Beg and Baltimore, 1996; Briscoe *et al*., 1996; Darnell, 1997; May and Ghosh, 1997). In cases where prolonged expression of a particular gene product induced by these activators is detrimental to the cell, it would be necessary to abolish their transcriptional activity almost immediately. A simple way to achieve this could be through the rapid proteasome-mediated degradation of these potent transcriptional activator proteins bound to their binding sites in the specific promoter. Recent evidence suggests that the intracellular levels of activated forms of steroid hormone-induced receptor proteins such as progesterone receptor, STATs and HSF-2 are indeed degraded rapidly by proteasomes (Chen *et al*., 1996; Kim and Maniatis, 1996; Mathew *et al*., 1998; Nawaz *et al*., 1999).

Earlier studies have suggested that potent acidic activators are toxic to eukaryotic cells perhaps because of their ability to trap GTFs in non-productive compartments in the cell and subsequently 'squelch' transcription of essential genes (Gill and Ptashne, 1988; Berger *et al*., 1990, 1992). Berger and co-workers (1992) have shown that the toxicity of GAL4VP16 in yeast cells can be alleviated by abolishing the function of either the GAL4 DNA-binding domain or the VP16 activation domain. Our data demonstrate that mutations that abolish the function of either one of these domains protect the activator from proteasome-mediated degradation. Taken together, these observations suggest that proteasome-mediated degradation of activator–target protein complexes could be the basis for cytotoxicity caused by the overexpression of potent activators. It is possible that prolonged activation of the proteasome-mediated protein degradation pathway in the nucleus triggered by the accumulation of stable activator–target protein complexes leads to the depletion of factors that are essential for transcription, and ultimately to cell death. Perhaps to avoid this potentially serious problem, many natural signal-responsive potent transcriptional activators remain in the cytoplasmic compartment and translocate to the nucleus to induce transcription of their target genes only for a brief period of time.

Materials and methods

Plasmids

All transcriptional activator fusion proteins described in this study were expressed from pCGNN (Attar and Gilman, 1992). Inserts cloned into pCGNN as *Xba*I–*Bam*HI fragments are transcribed under the control of the human cytomegalovirus (CMV) enhancer and promoter and are expressed with an N-terminal epitope tag (a 16-amino-acid portion of the *Haemophilus influenzae* hemagglutinin gene) and a nuclear localization sequence from the SV40 large T antigen. In some cases, activation domains were synthesized by PCR as fragments containing an *Xba*I site immediately upstream of the first codon, and a *Spe*I site, an in-frame stop codon and a *Bam*HI site immediately downstream of the last codon. Chimeric proteins comprising multiple components were assembled by stepwise insertion of *Xba*I–*Bam*HI fragments into *Spe*I–*Bam*HI. pCGNN-GAL4 plasmid was generated by ligating GAL4 coding sequences (amino acids 1–94) with *Xba*I- and *Bam*HI-digested vector. Activation domains from VP16 (amino acids 419–494), p65 (amino acids 450–550 and 361–550), SP1 (amino acids 263–291), p53 (amino acids 1–42), QIIIX18 (OCT-1), SRF (amino acids 412–508) and CTF (amino acids 399–499) were PCR amplified with appropriate primers, digested with *Xba*I–*Bam*HI and cloned into pCGNN-GAL4 expression vector. pCGNN-GAL4V8×2 was generated by inserting two copies of V8 domain-coding oligonucleotide sequences into *Spe*I- and *Bam*HI-digested pCGNN-GAL4 vector. pCGNN-GAL4V8×4, 6, 8 and 12 were generated by sequential addition of V8×2 oligonucletides into appropriate vectors. pCGNN-GAL4p65S and pCGNN-GAL4p65L were made by inserting p65 regions between amino acids 450 and 551, and 361 and 551, respectively, into *Spe*I and *Bam*HI-digested pCGNN-GAL4 vector. To make pCGNNGAL4p65LV8×8, the V8×8 fragment was excised from pCGNN-GAL4V8×8 vector and inserted between the *Spe*I and *Bam*HI sites of the pCGNN-GAL4p65L vector. pCGNN-GAL4V8×4(F-A) was generated by sequential insertion of V8(F-A)×2 oligonucleotides into *Spe*I- and *Bam*HI-digested pCGNN-GAL4 vector. pCGNN-GAL4V8×8(F-A) was made by inserting the V8×4(F-A) fragment between the *Spe*I and *Bam*HI sites of the pCGNN-GAL4V8×4(F-A) vector. pCGNN-GAL4M1V8×8 and pCGNN-GAL4M2V8×8 containing mutations in the GAL4 DNA-binding domain were made by site-directed mutagenesis of the template DNA derived from pCGNN-GAL4V8×8 vector.

Cell culture and stable cell lines

HT1080B cells were grown in minimal essential medium (MEM) supplemented with non-essential amino acids and 10% fetal bovine serum (FBS). To generate cells containing the pLH-5×GAL4-IL2-SEAP reporter stably integrated, helper-free retrovirus generated by conventional methods was used to infect HT1080 cells. Hundreds of hygromycin B- (300 mg/ml) resistant clones were pooled (HT1080B pool) and individual clones screened by transient transfection with pCG-GS. The most responsive clone, HT1080B, was selected for further analysis.

Transient transfections

HT1080 cells were grown at 37°C in MEM containing 10% FBS, non-essential amino acids and penicillin–streptomycin. At 24 h before transfection, ~2×10^5 cells were seeded in each well in a 12-well plate. Cells were transfected using Fugene as recommended (Boehringer Mannheim). In all cases, the total amount of DNA used in the transfections was adjusted to 2 μg/ml with pUC19. After transfection for 18 h, 100 μl of medium were removed and assayed for SEAP activity using a Luminescence Spectrometer (Perkin Elmer) at 350 nm excitation and 450 nm emission.

RNase protection assay

RNase protection assays were carried out essentially as previously described (Gilman, 1988).

Pulse–chase analysis

In all cases, ~18 h after transfection, HT1080B cells were washed twice in phosphate-buffered saline (PBS) and resuspended in MEM without methionine and cysteine (Gibco-BRL). The labeling mix containing 100 μCi/ml of [^{35}S]methionine and [^{35}S]cysteine was added to cells, and pulse labeling was carried out for either 1 or 2 h. The radioactive medium was then removed, and cells were washed twice with PBS and resuspended in the media containing 10% serum and 100 mM cold methionine and cysteine. After incubation in this medium for varying periods of time, cells were harvested, washed with ice-cold PBS twice and lysed in a buffer containing 10 mM Tris–HCl pH 7.4, 150 mM NaCl, 1% NP-40, 1 mM dithiothreitol (DTT) and 1 mM phenylmethylsulfonyl fluoride (PMSF). The HA-tagged activator proteins in the cell lysates were immunoprecipitated with anti-HA antibodies (Babco), washed extensively using the same buffer and fractionated by SDS–PAGE followed by autoradiography.

Western blotting

The medium was removed ~24 h after transfection and the cells were washed three times and harvested in PBS buffer. After a 10 s centrifugation, the cell pellet was resuspended in SDS sample buffer, boiled for 2 min and the samples were fractionated on 12% SDS–polyacrylamide gels. Western blotting with the indicated antibodies was carried out by following standard procedures.

Gel shift assay

^{32}P-labeled GAL4-binding site probes were prepared by the end-filling method using Klenow DNA polymerase. Nuclear extracts from transiently transfected cells were prepared as described previously (Natesan and Gilman, 1995). Appropriate amounts of nuclear extracts were incubated at room temperature for 15 min in a buffer containing 10 mM Tris–HCl pH 7.4, 60 mM NaCl, 5 mM $MgCl_2$, 1% bovine serum alumin (BSA), 10% glycerol, 1 mM DTT and 1 μg of poly(dI–dC) in 20 μl total volume. After the addition of the radioactively labeled probe, the reaction mix was incubated at room temperature for an additional 20 min. The samples were analyzed on 6% (39:1) polyacrylamide gels run in 0.5× Tris–borate–EDTA buffer.

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