Identification of a Region of p53 That Confers Lability*

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Degradation provides one means for controlling the cellular level of the p53 tumor suppressor. Here we have determined a structural element of p53 required for degradation. To create a substrate amenable to in vitro analysis of proteolysis, we appended to p53 the N terminus of antizyme, a protein that binds to and induces degradation of mammalian ornithine decarboxylase (ODC). We found using deletion analysis that an element within amino acids 100–150 is required for degradation of the fusion protein. A monoclonal antibody (PAb246) that binds close to this region prevents the degradation induced by human papillomavirus 16 E6 protein. Furthermore, we found that amino acids 100–150 of p53 can function as an independent domain to induce Trypanosoma brucei ODC, a stable protein, to be degraded in vivo or, by cooperating with an antizyme binding domain of ODC, to confer polyamine-dependent regulation.

Tumor suppressor p53 imposes negative regulation on the cell cycle, and abnormal inactivation of the protein has been shown to be related to cell transformation and tumorigenesis (1). Elimination of p53 function takes place by multiple means, including degradation, interaction with other proteins, interaction with mutant forms of p53 itself, or somatic or germine mutation (1). Degradation of p53 is influenced by protein-protein interactions. A variety of viral oncoproteins have been shown to act on p53. The association of p53 with SV40 large T antigen or with adenovirus 2 E1B-55K protein extends its shown to act on p53. The association of p53 with SV40 large T


determination of antizyme, a protein that binds to and induces degradation of mammalian ornithine decarboxylase (ODC). We found using deletion analysis that an element within amino acids 100–150 is required for degradation of the fusion protein. A monoclonal antibody (PAb246) that binds close to this region prevents the degradation induced by human papillomavirus 16 E6 protein. Furthermore, we found that amino acids 100–150 of p53 can function as an independent domain to induce Trypanosoma brucei ODC, a stable protein, to be degraded in vivo or, by cooperating with an antizyme binding domain of ODC, to confer polyamine-dependent regulation.

Degradation is mediated by the ATP-dependent and nonlysosomal large 26 S protease complex. Many short-lived proteins need to be modified by ubiquitination in order to be degraded (7–10), but some labile proteins such as ornithine decarboxylase (ODC) are not ubiquitinated (11–13). Ubiquitination is required for p53 degradation (5, 14). Studies from Howley and his colleagues (15) demonstrated that E6 and the associated host cell protein E6-AP act as a ubiquitin ligase E3, which leads to ubiquitination of p53 and its degradation. The structural elements of p53 needed for recognition and destruction by the proteolytic machinery remain unclear, as is true for most short-lived proteins. We propose that for the 26 S protease to act, substrate proteins must contain a structural motif, a degradation domain. This domain is required for proteins to undergo degradation via both ubiquitin-dependent and -independent pathways.

ODC, the key enzyme in the biosynthesis of polyamines, is a short-lived protein. Two types of degradation occur: polyamine-dependent and polyamine-independent (16). The C terminus of ODC suffices as a degradation domain to confer polyamine-independent degradation (16–18), but it is insufficient for polyamine-dependent degradation (16). Polyamines regulate ODC activity via induction of antizyme (19, 20). Antizyme binds to ODC, inhibits its activity, and promotes its degradation (16, 21, 22). Antizyme binding depends on an element near the N terminus of ODC (21). For polyamine-dependent degradation to occur, two elements are necessary within ODC: the antizyme binding domain and the C-terminal degradation domain (16). Furthermore, we showed that the C terminus of antizyme interacts with ODC and that the N-terminal half of antizyme is not involved in the antizyme/ODC interaction, but it is required for promoting ODC degradation (23). The N terminus of antizyme (NAZ), when directly coupled to diverse short-lived proteins, can direct their degradation (24). Because NAZ is a module that must function in collaboration with a degradation domain, a simple strategy can in principle be applied to identify such domains within a labile protein: appended NAZ and carry out deletion analysis.

Here we report that we have identified a degradation domain of p53 by fusing it to NAZ and making deletions within the p53 moiety. The degradation domain of p53 so identified was able to function, like the degradation domain of mouse ODC, to induce degradation of trypanosome ODC, a stable protein.

MATERIALS AND METHODS

In Vitro Transcription and Translation—Recombinant DNAs used for expression of fusion proteins by in vitro transcription and translation were made as follows. The DNA encoding each constituent protein fragment to be expressed was copied by PCR, using oligonucleotides that incorporated a common restriction endonuclease recognition site at the point of fusion. The PCR fragments encoding the protein regions to be fused were digested with the common restriction enzyme, ligated, and reamplified using the distal 5'- and 3'-oligonucleotides. The 5'-oligonucleotide used to copy the N-terminal element of each fusion contained a T7 RNA polymerase recognition site placed upstream of the translation initiation AUG codon. The resultant PCR product after the second round of amplification thus contained at the 5'-end a T7 RNA polymerase recognition site, followed by an open reading frame encoding the fusion protein.

In Vitro Degradation Assay—Recombinant DNA constructs produced by PCR as above were used as templates for in vitro transcription by T7 RNA polymerase. RNA was translated in vitro using a rabbit reticulocyte lysate (Promega) in the presence of [35S]methionine. The translated proteins were subjected to degradation in a rabbit reticulocyte lysate extract as described (16). Degradation was carried out at 30 °C, and the labeled protein remaining undegraded was examined by SDS-polyacrylamide gel electrophoresis and autoradiography. To inhibit degradation, incubation was carried out as above, except that 2 mM ATP-γS was used in place of ATP and the ATP-regenerating system was omitted.
examine the effect of monoclonal antibody on degradation, labeled mouse p53 was preincubated for 5 min on ice with 0.2 mg of PAb246 or PAb421 (Oncogene Science) before addition to the extract.

Transfection and in Vivo Degradation Assay—constructs TbODC-p53 100–150 and M314T-p53 100–150 were cloned into a mammalian expression vector under the control of the SV40 early promoter (25). The plasmids, along with pMC1 conferring neomycin/G418 resistance (26), were used to transfect mutant Chinese hamster ovary C55.7 cells devoid of endogenous ODC activity (27). Transformants were obtained by subjecting the cells simultaneously to two forms of selection: 1) with the drug G418, selective for cells expressing the neo gene encoded by pMC1, and 2) by incubation in polyamine-deficient medium, selective in the mutant ODC-deficient cells for expression of the transfected ODC gene, which is needed for polyamine biosynthesis. Pools of stably transformed clones were plated (approximately 106 cells/100-mm Falcon dish) and incubated at 37°C for 16 h. To elicit polyamine-mediated regulation and induce endogenous AZ, putrescine was added to the culture medium to a final concentration of 100 μM. ODC stability was assessed in some experiments by inhibiting protein synthesis with cycloheximide, 100 μg/ml of cell culture medium, and determining the rate of decay of enzymatic activity. Cell lysates were prepared after the indicated time of treatment and assayed for ODC activity as described (16, 28).

RESULTS

Identification of the p53 Degradation Domain—To determine if p53 has a domain that can collaborate with NAZ, we fused NAZ directly to p53 and analyzed the lability of NAZ-p53, as well as a series of deletions within the 393-amino acid p53 moiety of the NAZ-p53 parent molecule. Deletions were made from each end or from both ends (Fig. 1). We found that NAZ-p53 1–202 was very extensively degraded and that deleting 99 amino acids from its C terminus (NAZ-p53 1–103) greatly stabilized it (Fig. 2A). Degradation of NAZ-p53 1–202 is more extensive than that of NAZ-p53, the parent molecule, or of NAZ-p53 1–304. This could be because either 1) amino acids 203–304 protect 103–202, perhaps by altering its structure or by denying access to a proteolytic process, or 2) the degradation domain in amino acids 103–202 needs, like the degradation domain of mouse ODC, to be topologically at or close to the C terminus of the fusion protein (16). These results suggested that amino acids 101–202 contain a degradation domain. A more refined deletion from the C terminus of p53 demonstrated that removal of amino acids 151–202 from NAZ-p53 1–202 (to form NAZ-p53 1–150) did not interfere with degradation (Fig. 2D), but the internal deletion of amino acids 101–139 from NAZ-p53 1–200 (to form NAZ-p53 1–200 A101–139) prevented degradation (Fig. 2D). Therefore, we conclude that amino acids 100–150 contain a degradation domain of p53 that is active in an NAZ fusion.

NAZ-mediated Degradation of p531–150 Is Ubiquitin-independent—By grafting NAZ to p53, we created a fusion protein that is labile in vitro and used deletion analysis to identify a degradation-determining region within p53. The NAZ-p53 fusion protein could in principle undergo degradation via a ubiquitin-intervened intermediate, as is normal for p53, or by a ubiquitin-independent pathway, as in the case for ODC when it is associated with antizyme. To examine this, we carried out in vitro degradation in the presence of ATP-γS, which has been shown to block degradation but not ubiquitination (5). As a positive control, we used HPV16 E6-mediated degradation of p53, which is ubiquitin-dependent. In the presence of E6 and ATP, p53 was degraded (Fig. 3). When ATP-γS was used in place of ATP, degradation was blocked and high molecular weight forms of p53 accumulated. NAZ-p531–150 also was degraded in the presence of ATP. Substitution of ATP-γS for ATP blocked degradation here as well, but no high molecular mass form of the NAZ-p53 1–150 fusion protein appeared. Therefore, NAZ-induced degradation is ubiquitin-independent. The N terminus of antizyme seems to bypass the modification normally required for p53 degradation and imposes instead the properties associated with degradation of ODC.

A Monoclonal Antibody Blocks E6-mediated p53 Degradation—Next we asked if the degradation domain, identified within the fusion proteins, is instead involved in degradation of p53 itself. To test this, we used a monoclonal antibody (PAb246) that specifically recognizes mouse p53 amino acids 88–109. We determined whether it can block E6-mediated p53 degradation. Because there is no cross-reaction of the antibody with human p53 and because similarly directed antibodies to the human protein were not available, we used mouse p53 in the study. PAb246 recognizes the native conformation of its epitope (29). Mouse p53 degradation was induced by human E6, but the presence of the antibody prevented this degradation (Fig. 4). A control monoclonal antibody (PAb421) against the C terminus of p53 (30), however, had no effect on degradation. These results strongly suggest that the region of p53 identified as necessary for degradation must be accessible for that process to occur and that antibody can...
The Degradation Domain of p53 Induces Trypanosome ODC Degradation—We have previously shown that the mouse ODC C terminus is required for its in vivo degradation and that it confers lability on the homologous but stable ODC (TbODC) found in Trypanosoma brucei, the agent of African sleeping sickness (16, 17, 28, 31). We asked whether amino acids 100–150 of p53 containing the degradation domain can act in place of the mouse ODC C terminus. This region was fused to the C terminus of TbODC to make TbODC-p53 100–150. We stably transfected C55.7 ODC-deficient Chinese hamster ovary cells with TbODC-p53 100–150. We determined the stability of the fusion protein by measuring the rate at which ODC activity, provided by the fusion protein, declined in cells treated with cycloheximide to inhibit protein synthesis. Measurement of ODC activity provides an accurate surrogate determination of its protein level (32). ODC activity fell with a half-life of about 30 min (Fig. 5). In contrast, the ODC activity provided by a construct encoding TbODC without the p53 degradation domain remained unaffected by cycloheximide treatment. This result shows that amino acids 100–150 of p53 can confer in vivo lability on the stable protein TbODC.

The Degradation Domain of p53 Plus the Antizyme Binding Domain Confers Polyamine Regulation on Trypanosome ODC—To determine whether the degradation domain of p53 can cooperate with the AZ binding domain to subject TbODC to polyamine-mediated degradation, we provided a binding site for AZ. The N terminus of the construct TbODC-p53 100–150 was replaced with an equivalent region of mouse ODC containing the AZ binding domain to form M314T-p53 100–150. Cells were transformed as above and treated with the polyamine precursor putrescine for the indicated times to induce AZ. ODC activity in the form of M314T-p53 100–150 was regulated by polyamines (Fig. 6). ODC declined by about 65% from control values within 4 h of treatment and subsequently remained at this level. However, M314Tb, identical to M314Tb-p53 100–150 but for the absence of the degradation domain of p53, was insensitive to regulation by polyamines. Therefore, p53 in this context can provide the same functional properties as the C-terminal degradation domain of ODC.
Identification of a Region of p53 That Confers Lability

p53 is among the most labile of proteins. Its degradation is required for cells to enter S phase. Overexpression of the functioning protein or reduced degradation of the protein via DNA damage arrests cell cycle progression at the G1/S boundary (33, 34). Ubiquitination has been demonstrated both in vivo and in vitro to be associated with p53 degradation (5, 14). In ts 20 cells, with a temperature-sensitive mutation of ubiquitin-activating enzyme (E1), degradation of p53 is blocked at the non-permissive temperature (14). Howley and his colleagues (35, 36) have shown that HPV16 E6 protein activates p53 degradation in vitro, and the activation requires its associated protein, E6-AP, a ubiquitous host cell protein co-factor. Their recent studies have indicated that these two proteins, E6 and E6-AP, together function as a ubiquitin-protein ligase (15). In addition, E6-mediated p53 degradation involves a novel ubiquitin carrier protein (E2) for p53 ubiquitination (15, 37, 38). This E2 is distinguishable by its chromatographic properties from those previously characterized. The novel E2 may participate in the ubiquitination of multiple endogenous substrates.

By utilizing the NAZ-p53 protein, we carried out deletion analysis of the p53 moiety and found such constructs to be labile only if they contain amino acids 100–150 of p53. Having identified amino acids 100–150 as a putative degradation domain in p53, we tested its modularity by placing it in an ODC context. It was able to destabilize trypanosome ODC, as does the degradation domain of mouse ODC. Last, we showed that in mouse p53, E6-mediated degradation could be blocked by an antibody specific for this region. Except for the degradation domain here identified, the structural determinants of p53 degradation such as the binding site for the E6E6-AP complex and the ubiquitinated lysine(s) have not determined. Such information will facilitate our understanding of p53 degradation.

Recent x-ray crystallographic studies (39) of a complex of p53 core and DNA indicate that the protease-resistant core (amino acids 94–312) consists of a β sandwich hydrophobic core and a loop-sheet-helix motif. A conserved region in amino acids 117–142 contains part of the L1 loop, the paired β S2-S2’ sheets and their short connecting loop, and a part of β strand 3. The back of the β S2-S2’ hairpin interacts with a pocket formed by the S1, S3, and S8 β sheets, and the pocket leaves the hairpin buried. We hypothesize that the S2-S2’ hairpin may constitute an important part of the degradation domain and that ubiquitination exposes the buried structure. The PAb246 may stabilize a native conformation, preventing exposure of the structure and thereby blocking E6-mediated p53 degradation. Deletion of amino acids 203–304 from NAZ-p53 1–304 (to make NAZ-p53 1–202) destroys the pocket, which can be functionally accessible for the degradation machinery by NAZ. This could also explain the increased lability of NAZ-p53 1–202 compared with NAZ-p53 1–304.

Assignment of the degradation domain in p53 to amino acids 100–150 may illuminate two observations. First it could explain regulation of the half-life of p53 by large T antigen of the SV40 virus. The half-life of p53 protein is regulated by its associated viral oncoproteins. Among the oncoproteins, association with the SV40 large T antigen has been shown to extend the p53 half-life. The minimal binding region of p53 for large T antigen targeting has been identified as amino acids 94–293 (40). The degradation domain of p53, which we have identified here, is located within this binding region. Therefore, the association of p53 with large T antigen could cover the degradation domain and prevent p53 degradation. Second, our observations are consistent with the distribution of mutations that increase the half-life of p53. Mutations in p53 are commonly found in tumors, and the mutated protein often has a much longer half-life than wild type p53 (6). The mutations are widely distributed within the protein but are mainly located in four regions (41). One of the regions lies in the amino acid 100–150 degradation domain. Mutations in the region may either directly change the degradation domain itself to prevent protease targeting or change protein conformation to prevent ubiquitination. Although we have experimentally tested this, we speculate that most p53 mutations outside the degradation domain extend half-life by reducing the efficiency of ubiquitination. Conversely, we expect mutations in the 100–150 region to alter stability without preventing ubiquitination. One such temperature-sensitive mutation, murine p53 A135V, originally found in a mouse tumor, appears to have that property (37). It is important to remember that p53 mutations that cause tumors are likely to constitute a highly unrepresentative subset of those that can stabilize; because p53 is a tumor suppressor, oncogenic mutations that stabilize must also act as dominant negative mutations. Site-directed deletion and substitution mutations within the amino acid 100–150 region are therefore required to test these predictions without selection bias.

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