MOLECULAR AND CELLULAR BIOLOGY, Jan. 1997, p. 460–468 Vol. 17, No. 1
0270-7306/97/$04.00+0

Proteolytic Cleavage of Human p53 by Calpain: a Potential Regulator of Protein Stability

MICHAEL H. G. KUBBUTAT AND KAREN H. VOUSDEN*

ABL Basic Research Program, National Cancer Institute-Frederick Cancer Research and Development Center, Frederick, Maryland 21702

Received 14 August 1996/Returned for modification 30 September 1996/Accepted 11 October 1996

The p53 tumor suppressor protein is activated in cells in response to DNA damage and prevents the replication of cells sustaining genetic damage by inducing a cell cycle arrest or apoptosis. Activation of p53 is accompanied by stabilization of the protein, resulting in accumulation to high levels within the cell. p53 is normally degraded through the proteasome following ubiquitination, although the mechanisms which regulate this proteolysis in normal cells and how the p53 protein becomes stabilized following DNA damage are not well understood. We show here that p53 can also be a substrate for cleavage by the calcium-activated neutral protease, calpain, and that a preferential site for calpain cleavage exists within the N terminus of the p53 protein. Treatment of cells expressing wild-type p53 with an inhibitor of calpain resulted in the stabilization of the p53 protein. By contrast, in vitro or in vivo degradation mediated by human papillomavirus E6 protein was unaffected by the calpain inhibitor, indicating that the stabilization did not result from inhibition of the proteasome. These results suggest that calpain cleavage plays a role in regulating p53 stability.

The p53 protein plays an essential role in protection from malignant development, and mutational loss of p53 function is the most frequently detected genetic event in human cancers (31). Although normal development can occur in the absence of p53, loss of p53 function results in elevated rates of tumor development, suggesting that p53 functions primarily as a checkpoint to prevent abnormal cell proliferation (17, 33). p53 activity has been implicated in numerous processes, including regulation of centrosome duplication (21) and spindle checkpoints (14), differentiation (1, 18), senescence (2) and regulation of angiogenesis (15). The best-understood functions of p53 are the activation of a G_1 cell cycle arrest and participation in programmed cell death, or apoptosis (reviewed in reference 5), although many of the p53 activities described could play a role in tumor suppression. The p53 protein functions as a sequence-specific activator of transcription (22, 35, 60), and many cellular genes regulated by p53-responsive promoters have been described. Although there are clearly additional transcriptionally independent activities of p53 which contribute to at least some functions, such as apoptosis (6, 28, 63), in many cells the ability of p53 to function as a transcription factor appears to be an integral part of its ability to suppress tumor cell growth (11, 50, 52, 67).

In most normal cells the p53 protein is expressed at very low levels due to the relatively short half-life of the protein. Accumulation of the p53 protein occurs following DNA damage (43), and activation of p53 is also apparent following other types of stress, such as growth arrest (69), hypoxia (25), or ribonucleotide depletion (40). The DNA damage-induced accumulation of p53 is principally the result of the stabilization of the p53 protein (20, 34), although transcriptional and translational regulation may also occur. Elevation of protein levels appears to be one component of the p53 response, although it is not essential for activation of p53 function (32). It seems likely that the normal p53 response involves both activation of a latent form of the protein, possibly by phosphorylation or redox regulation, and enhanced stability to increase the amount of p53 in the cell.

Despite significant advances in our understanding of how p53 mediates downstream effects such as cell cycle arrest and apoptosis, relatively little is known about how the stability of the protein is regulated in normal cells and how this stability is enhanced following DNA damage. There is evidence that p53 can be degraded through ubiquitin-dependent proteolysis (42), and cells which are defective for this proteasome-dependent pathway show elevated levels of p53 (9). The role of ubiquitin-mediated degradation is most clearly seen following the interaction of p53 with the E6 protein encoded by the human papillomaviruses (HPV) associated with cervical malignancies. E6, in association with the cell protein E6-AP, can function as a ubiquitin ligase (54) which targets p53 for degradation (56). Cells expressing E6 fail to stabilize p53 in response to DNA damage and cannot activate the normal p53-dependent responses (36). Whether E6 enhances the normal mechanism of p53 degradation or whether a different proteolytic pathway is utilized is not clear, although a cellular homolog for the function of E6 has not been described. Mutant p53 proteins expressed in tumor cells are frequently found at very high levels due to increased stability (68) which appears to result from an alteration in cellular environment rather than resistance to normal degradation by the mutant p53 protein (62). Indeed, several p53 mutants which show enhanced stability in tumor cells are targeted for degradation by E6 as efficiently as the wild-type protein (13, 55).

The nonlysosomal calcium-activated neutral protease, or calpain (EC 3.4.22.17), is present in almost all cells, and at least two isoforms of the enzyme, μ- and *m*-calpain, which differ mainly in the calcium concentration needed for their activation in vitro, exist. Calpain belongs to the family of cysteine proteases and consists of an 80-kDa catalytic subunit and a smaller 30-kDa subunit which is identical in all isoforms. Both subunits can bind calcium, but in addition calpain activity can also be regulated by autoproteolysis and the inhibitor protein calpastatin (23), suggesting that, like the proteasome, calpains are part of a regulatory proteolytic system. The exact function of cal-

* Corresponding author. Mailing address: ABL Basic Research Program, NCI-FCRDC, Building 560, Room 22-96, West 7th St., Frederick, MD 21702-1201. Phone: (301) 846-1726. Fax: (301) 846-1666. E-mail: vousden@ncifcrf.gov.

pain is still obscure, but a role of calpains in platelet aggregation, neuronal long-term potentiation, neutrophil activation (reviewed in reference 10), oocyte maturation (64), and apoptosis (53, 58, 70) has been suggested, and it has been shown that calpain can cleave talin, filamin, fodrin, protein kinase C (10), c-mos (64), and several transcription factors, such as c-jun and c-fos (30, 65).

In this study we demonstrate proteolytic cleavage of p53 by an activity in cell lysates which we identified as calpain. The preferential site for cleavage is at the N terminus of the p53 protein, and inhibition of calpain cleavage correlated with enhanced stability of the p53 protein.

MATERIALS AND METHODS

Cell culture. MCF-7 (breast carcinoma, p53 wild type), C33a (HPV-negative cervical carcinoma, mutant p53), RKO (colon cancer, p53 wild type), and RKO/16E6 cells were maintained in Dulbecco modified Eagle medium–10% fetal calf serum.

Plasmids and in vitro translation. Plasmid pProSp53 encoding human wild-type p53 (47); plasmids encoding mutant p53 protein, pGEM3Z 232Thr, pGEM3Z 245Val (13), pGEM4Z ΔI (45), and pRC/CMV 22Gln/23Ser (39); and pGEM HPV16 E6 encoding HPV type (HPV16) E6 protein (12) have been described previously. Plasmids encoding mutants p53 15Ala, p53 15Asp, p53 Δ28-40, and p53 Δ41-49 were constructed by site-directed mutagenesis and subcloned into pGEM4Z (44). In vitro translation of human p53 proteins and HPV16 E6 proteins in the presence of [^{35}S]methionine was performed in rabbit reticulocyte lysate or wheat germ extract as described elsewhere (45).

Inhibitors and purified calpain. Leupeptin, E64, calpain inhibitor I, adenosine-5′-*O*-C3-thiotriphosphate (ATPγS), and adenylyl-imidodiphosphate (AMP-PNP) were purchased from Boehringer (Indianapolis, Ind.). Calpastatin peptide and phenylmethylsulfonyl fluoride (PMSF) were from Sigma (St. Louis, Mo.), and the inhibitor of interleukin 1β-converting enzyme (ICE)-like proteases YVAD-cmk was from Bachem Bioscience (King of Prussia, Pa.). The purified 80-kDa subunit of rabbit *m*-calpain was obtained from Sigma.

Monoclonal antibodies. Monoclonal antibodies DO-1 (Ab-6), PAb421 (Ab-1), PAb1620 (Ab-5), PAb1801 (Ab-2), and PAb240 (Ab-3) against p53 and anti-c-jun (Ab-1) were purchased from Oncogene Science (Cambridge, Mass.). Monoclonal antibody against the retinoblastoma gene product (Rb) (clone G3-245) was from Pharmingen (San Diego, Calif.). The anti-Raf-1 antibody was kindly provided by D. Morrison (National Cancer Institute, Frederick, Md.).

Cell lysate, nuclear extract, and cytoplasmic fraction. Cell lysates were prepared freshly in ice cold Nonidet P-40 buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 0.1% Nonidet P-40, 1 mM dithiothreitol, 1 mM PMSF) and rotated for 30 min at 4°C. Cell debris was pelleted by centrifugation (15 min, 13,000 × *g*, 4°C), and the supernatant was used in the p53 cleavage assay.

Nuclear extract and cytoplasmic fraction from MCF-7 cells were prepared as previously described (16, 57). To verify the purity of the preparation, aliquots of nuclear extract and cytoplasmic fraction were separated by sodium dodecyl sulfate–12% polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a nitrocellulose membrane. The membrane was first probed with a monoclonal antibody against Rb as a nuclear marker protein, stripped, and then reprobed with a monoclonal antibody against Raf-1 as a cytoplasmic marker protein.

Cleavage assay. Either in vitro-translated ^{35}S-labelled protein (5 μl) or C33a cell lysate (5 μl) was mixed on ice with cleavage buffer (25 mM Tris-HCl [pH 7.5], 100 mM NaCl, 3 mM dithiothreitol), and, if required, protease inhibitors or antibodies were added. In cleavage experiments including antibodies (1 μg/50 μl), this mixture was preincubated on ice for 30 min. Following addition of 10 μl of MCF-7 cell lysate or the amount of nuclear extract or cytoplasmic fraction indicated in the figure legends and incubation at room temperature, aliquots were taken at the time points indicated in the figures and subjected to SDS–15% PAGE. Either the gels were dried and evaluated by autoradiography or proteins were transferred onto nitrocellulose membranes by Western blotting and the membranes were probed with anti-p53 antibodies DO-1 and PAb1801. p53 protein was visualized by enhanced chemiluminescence (Amersham, Arlington Heights, Ill.).

Cleavage assays using purified *m*-calpain (4 U/ml) were carried out in phosphate-buffered saline supplemented with 1 mM $CaCl_2$ at room temperature as described in reference 7. HPV16 E6 protein-mediated in vitro degradation of p53 was performed in the presence of rabbit reticulocyte lysate as previously described (12).

Measurement of p53 half-life. Logarithmically growing cells were preincubated for 1 h in the presence or absence of calpain inhibitor I (16 μM). Incubation with medium lacking methionine and cysteine for 30 min was followed by a 1-h pulse using medium containing 100 μCi of ^{35}S-labelled methionine and ^{35}S-labelled cysteine (ProMix; Amersham). Cells were washed in phosphate-buffered saline and incubated in medium containing an excess of cold methionine and cysteine for the times indicated in the figures. Cells which were preincubated with calpain inhibitor I were continuously treated with the inhibitor. p53 protein was immunoprecipitated from cell lysates with PAb 421 as previously described (46), separated by SDS-PAGE, and detected by autoradiography.

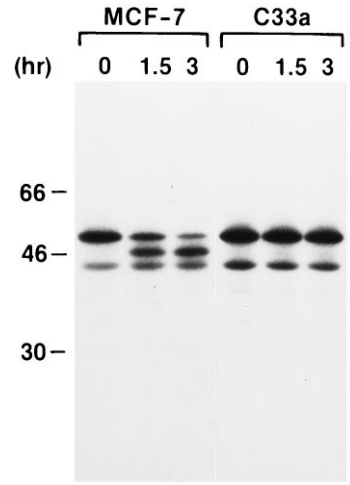

FIG. 1. Cleavage of p53 by MCF-7 lysate. Radioactively labelled in vitro-translated p53 protein was incubated at room temperature with lysates of MCF-7 and C33a cells. Aliquots were taken at the indicated time points, subjected to SDS-PAGE, and evaluated by autoradiography. Sizes are given on the left in kilodaltons.

Immunofluorescence. MCF-7 cells were treated for 16 h with actinomycin D or 3 h with 16 μM calpain inhibitor I or were left untreated, and immunofluorescence staining was performed as described previously (41) using monoclonal antibody DO-1. DNA was labelled by incubation with 4,6-diamidino-2-phenylindole (DAPI).

RESULTS

Calpain cleaves p53. Previous in vitro studies which have shown efficient ubiquitin-dependent degradation of p53 in rabbit reticulocyte lysate in the presence of HPV E6 have also demonstrated the relative stability of p53 under the same assay conditions in the absence of the E6 protein (12, 56), suggesting that at least one of the components normally regulating p53 proteolysis is missing. In an attempt to reconstitute p53 degradation in vitro, we supplemented p53 protein translated in rabbit reticulocyte lysate with extracts made from MCF-7 cells, a cell line which expresses wild-type p53 which can be stabilized following DNA damage (29). Although we were unable to detect a component in the cell lysate which could substitute for E6 in mediating ubiquitin-dependent proteolysis of the p53 protein, we were able to detect cleavage of the full-length p53 resulting in the generation of a smaller protein of 46 kDa (Fig. 1). This cleavage activity was not detected in C33a cells, a cell line expressing high levels of mutant p53 (66).

Several protease inhibitors were examined to determine the identity of the proteolytic activity in MCF-7 cells. No evidence of ubiquitinated p53 was found, and we were able to confirm that the proteolysis observed was not related to proteasome-associated degradation by demonstrating independence of ATP. Incubation with two ATP inhibitors, AMP-PNP and ATPγS (Fig. 2A), or preincubation with apyrase (data not shown) failed to inhibit the cleavage of p53 to generate the 46-kDa product. The cleavage was inhibited, however, by EDTA (Fig. 2A), and closer analysis confirmed that the cleavage was calcium dependent. Inhibition of the p53 cleavage by EGTA was clearly relieved by the addition of excess $CaCl_2$ but unaffected by $MgCl_2$ (Fig. 2B). A candidate ATP-independent, calcium-dependent protease is calpain, and this was tested directly by examining inhibition of the p53 cleavage activity by

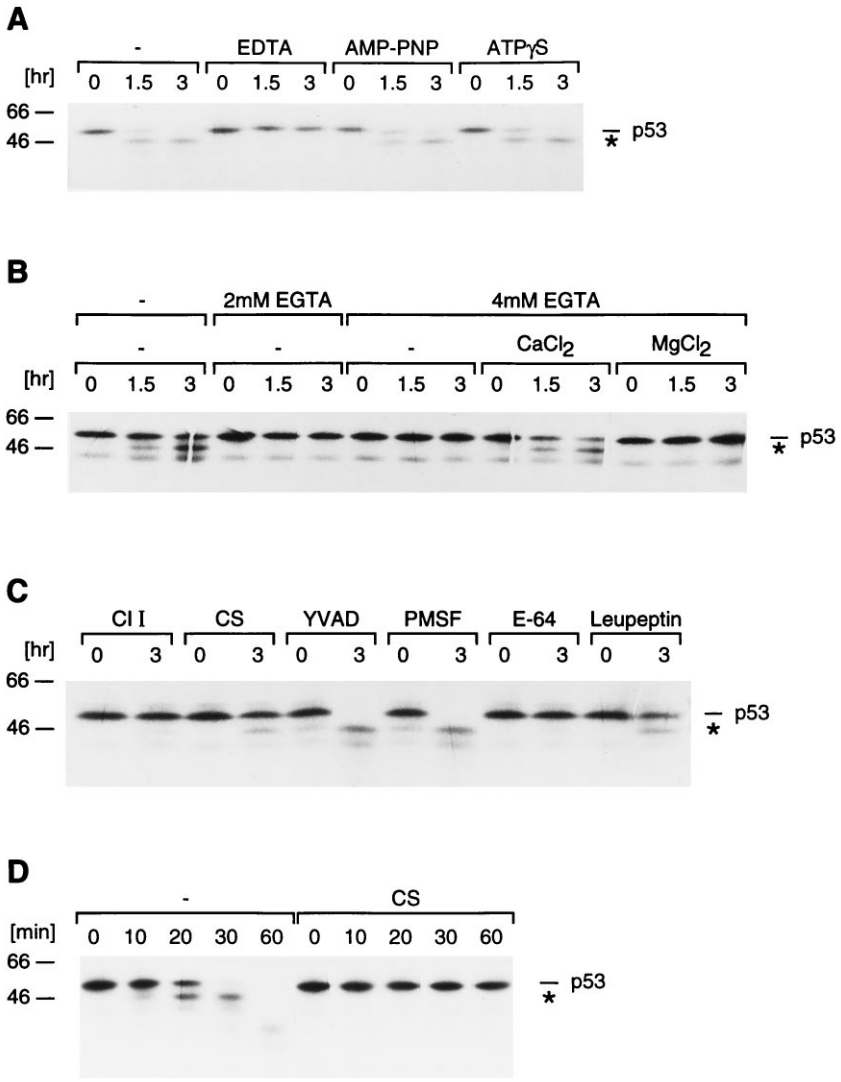

FIG. 2. (A) Effects of EDTA (2 mM), ATPγS (4 mM), and AMP-PNP (4 mM) on cleavage of p53 by lysate of MCF-7 cells. (B) Calcium (20 mM), but not magnesium (200 mM), can overcome EGTA-mediated inhibition of p53 cleavage by lysate of MCF-7 cells. (C) Effects of various protease inhibitors on cleavage of p53 by lysate of MCF-7 cells. Abbreviations: CI I, calpain inhibitor I (45 μM); CS, calpastatin peptide (30 μg/ml); YVAD, YVAD-cmk (100 μM). Other inhibitors: PMSF, 2 mM; E-64, 50 μM; leupeptin, 10 μM. (D) Purified calpain cleaves in vitro-translated p53 in vitro (CS, calpastatin peptide, 100 μg/ml). Radioactively labelled in vitro-translated p53 protein was incubated at room temperature with lysate of MCF-7 cells or purified calpain (4 U/ml) in the presence or absence (−) of the indicated amount of inhibitor and/or salt. Aliquots were taken at the indicated time points, subjected to SDS-PAGE, and evaluated by autoradiography. The positions of full-length p53 and the cleavage product (*) are indicated. Sizes are given on the left in kilodaltons.

calpain inhibitors (Fig. 2C). Although the cleavage was unaffected by the addition of PMSF (an inhibitor of serine protease) or YVAD-cmk (a specific inhibitor of ICE-like proteases), loss of cleavage activity following the addition of an inhibitor of cysteine proteases (E-64) and more specific inhibitors of calpain (leupeptin, calpain inhibitor I, and calpastatin peptide) confirmed the identity of the protease in the MCF-7 cell extract as calpain. Interestingly, although calpain is expressed in most cells, we were frequently unable to detect the cleavage activity when testing lysates from different cell types (e.g., C33a [Fig. 1]). This did not correlate with p53 levels and is probably due to activity of the endogenous calpain inhibitor, calpastatin, during the preparation of the lysates. Purified rabbit calpain was used in place of the MCF-7 cell lysate and a similar cleavage of p53 was seen (Fig. 2D), although prolonged incubation resulted in further degradation of the primary 46-kDa cleavage product, an activity which was also sometimes seen following prolonged incubation with MCF-7 cell lysate (see Fig. 3C).

Calpain preferentially cleaves in the N terminus of p53. Cleavage of the p53 protein by calpain to a distinct 46-kDa form suggested the presence of a single preferential cleavage site. In vitro translation of p53 frequently gives rise to two translation products, the full-length protein and a smaller protein of about 42 kDa which is likely to be the product of internal initiation. Since this smaller translation product appeared to be resistant to cleavage in this assay (Fig. 1), we investigated the possibility that a preferred cleavage site for calpain resides within the N terminus of p53. Analysis of a series of p53 mutants showed that point mutants within the DNA binding domain of the protein (232Thr and 245Val), such as those commonly found in human tumors, did not affect

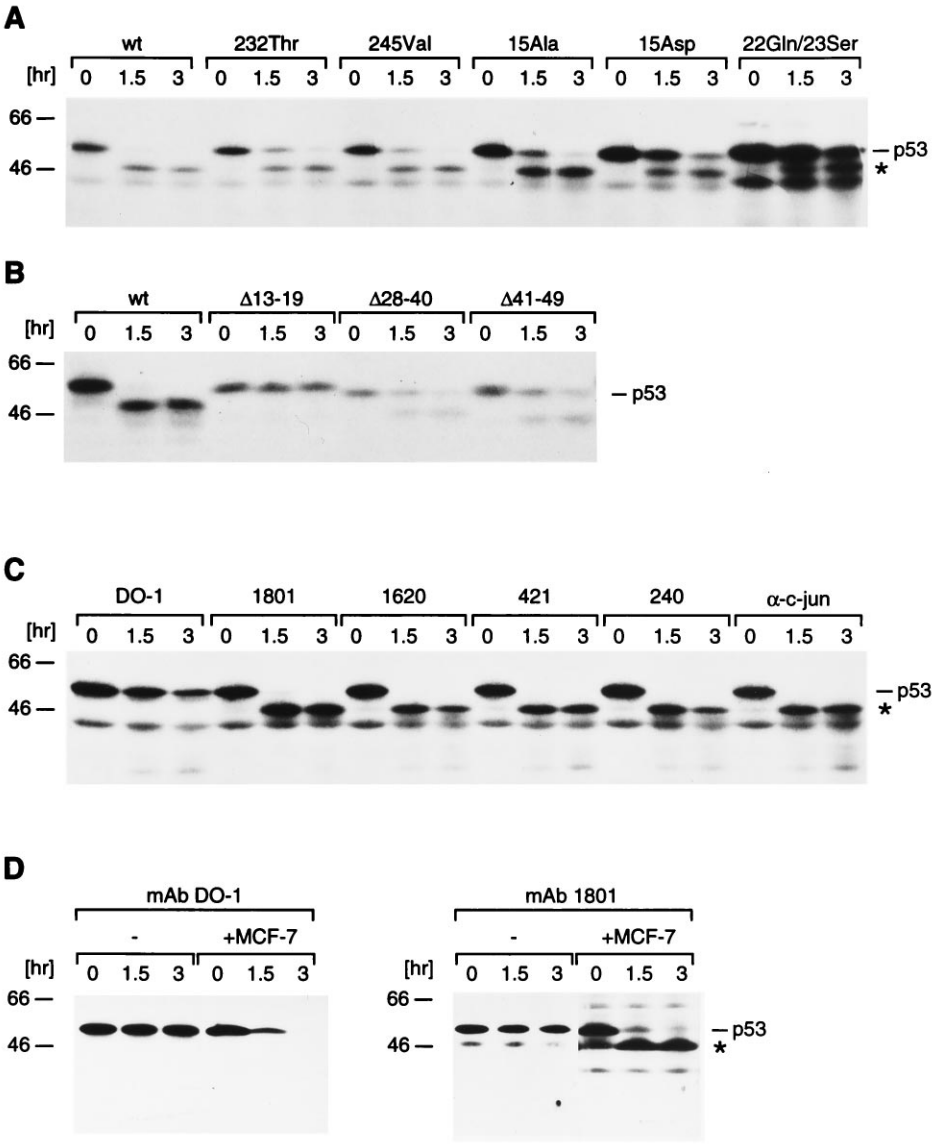

FIG. 3. Analysis of different p53 point (A) and deletion (B) mutants in cleavage assay using lysate of MCF-7 cells (wt, wild type). (C) Effects of different anti-p53 antibodies on cleavage of p53 by MCF-7 cell lysate. In vitro-translated p53 protein was preincubated on ice with monoclonal antibodies (20 μg/ml), mixed with lysate of MCF-7 cells, and further incubated at room temperature. (D) Western blot analysis for p53 protein in C33a lysate after incubation with lysate from MCF-7 cells. Lysate of C33a cells containing a high level of mutant p53 was incubated at room temperature in the presence or absence (−) of MCF-7 cell lysate. Aliquots taken at different time points were probed for p53 protein by using monoclonal antibodies (mAb) DO-1 and PAb1801. The positions of full-length p53 and the cleavage product (*) are indicated. Sizes are given on the left in kilodaltons.

the sensitivity to calpain cleavage (Fig. 3A). The mutant protein expressed in C33a cells (273Cys) also retained sensitivity to calpain cleavage (Fig. 3D). Mutations altering a phosphorylated serine at amino acid 15, a potential target for double-stranded-DNA-dependent protein kinase, to either alanine or aspartic acid (15Ala and 15Asp) also failed to affect cleavage. However, a p53 protein carrying a double point mutation within the transactivation domain (22Gln/23Ser) showed some resistance to the cleavage. Further analysis of p53 deletion mutants showed that loss of amino acids 13 to 19 (Δ13-19), encompassing conserved region I, rendered the p53 protein completely resistant to calpain cleavage (Fig. 3B), suggesting that the cleavage or recognition site for the protease must lie within this region of p53. Deletion of residues 28 to 40 or 41 to 49 (Δ28-40 and Δ41-49) did not affect calpain cleavage.

In order to confirm that the calpain cleavage site was localized to this region of the p53 protein, the effect of preincubating the p53 protein with various monoclonal antibodies was examined (Fig. 3C). The epitope recognized by the anti-p53 monoclonal antibody DO-1 has been identified as p53 residues 20 to 25 (59), and previous studies have shown that the p53ΔI mutant is very inefficiently recognized by this antibody (45). Several other antibodies with epitopes distant from the proposed cleavage site were also used, including PAb1801 (recognizing an epitope encompassing residues 46 to 55), PAb240 (recognizing an epitope encompassing residues 211 to 220), PAb421 (recognizing an epitope encompassing residues 371 to 380), and PAb1620 (recognizing a nonlinear, conformational epitope) (38). Binding of an antibody at the DO-1 epitope efficiently blocked the calpain-dependent cleavage of p53, al-

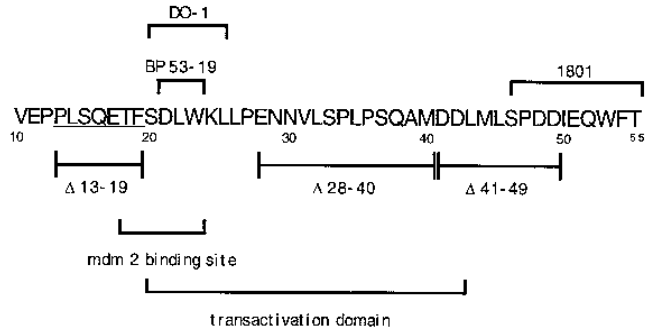

FIG. 4. N-terminal partial amino acid sequence of human p53 protein. Underlined amino acid residues (13 to 19) represent the conserved box I. Marked are the epitopes of antibodies DO-1, BP53-19, and PAb1801; the mdm2 binding site; the transactivation domain; and the deletion mutants used in this study.

though this was unaffected by any of the other p53-specific antibodies or a control antibody which recognizes c-jun (Fig. 3C).

The extent of the N-terminal cleavage was then analyzed by examining the reactivity of the 46-kDa cleavage product with p53-specific monoclonal antibodies. Full-length p53 is recognized by both DO-1 (epitope 20–25) and PAb1801 (epitope 46–55) (Fig. 3D). Although there is some cross-reactivity between PAb1801 and a nonspecific band at around 46 kDa, it is clear that while the appearance of the 46-kDa cleavage product can be detected by PAb1801, only loss of the full-length protein following the calpain cleavage reaction can be detected with DO-1. The inability of DO-1 to interact with the cleavage product is mirrored by a similar lack of reactivity between the cleavage product and the p53 antibody BP53-19 (4), which recognizes a smaller epitope encompassing residues 21 to 23 (59) (data not shown). In summary (Fig. 4), it seems likely that deletion of conserved region I (residues 13 to 19) interferes with calpain recognition and that the cleavage position occurs within the DO-1 epitope (residues 20 to 25). Normal cleavage of the deletion mutant Δ28-40 delineates the C-terminal boundary of this site.

Biological relevance of calpain cleavage. Calpain has been localized to the cytoplasm (10) and nucleus of cells (24, 49), and since p53 is predominantly a nuclear protein, we analyzed nuclear and cytoplasmic fractions from MCF-7 cells for p53 cleavage activity (Fig. 5). The purity of each fraction was assessed by detection of a known cytoplasmic protein (Raf-1) and a known nuclear protein (Rb) (Fig. 5B). These analyses revealed a slight nuclear contamination in the cytoplasmic fraction but suggested that the nuclear extract was essentially free from cytoplasmic contamination. Analysis of the cleavage activity in these extracts showed that active calpain could be detected in both cytoplasmic and nuclear extracts, indicating that both p53 and the protease are present in the same cellular compartment (Fig. 5A).

Although clearly evident in the in vitro assays, we were unable to detect the 46-kDa p53 cleavage product in lysates from MCF-7 and RKO cells, suggesting either that this proteolytic cleavage does not occur in vivo or that the cleavage product is unstable in cells. To test this directly, we examined the p53 levels in MCF-7 cells following incubation with calpain inhibitor I (Fig. 6). Compared to levels in untreated cells (Fig. 6A and B), most of the cells showed elevated p53 levels following treatment with calpain inhibitor I (Fig. 6E and F) similar to those seen following DNA damage induced by actinomycin D (Fig. 6C and D). Increased p53 levels were also seen in these cells following treatment with calpain inhibitor II, although higher concentrations of this inhibitor (100 to 200 μM) were necessary (data not shown). Since calpain inhibitor I is also a weak inhibitor of ubiquitin-dependent degradation (19, 51), we sought to distinguish between interference with ubiquitin-dependent and calpain-dependent proteolysis by examining the effect of calpain inhibitor I on the ubiquitin-dependent degradation of p53 by E6, a reaction which is clearly calpain independent. RKO cells, which express wild-type p53, and RKO cells expressing HPV16 E6 (36) were examined following treatment with calpain inhibitor I (Fig. 7A), and

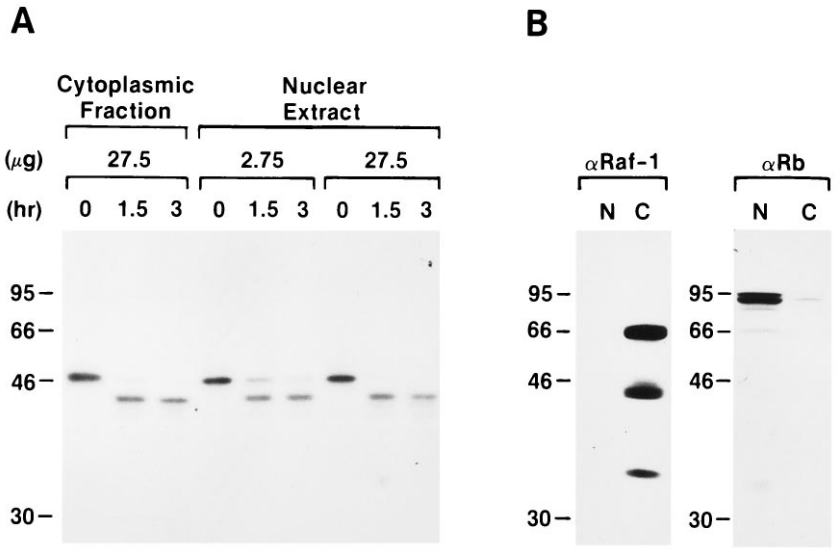

FIG. 5. Proteolytic activity that cleaves p53 protein can be found in nuclear extract and cytoplasmic fraction of MCF-7 cells. In vitro-translated p53 protein was incubated with the indicated amounts of nuclear extract and cytoplasmic fraction, and aliquots were subjected to SDS-PAGE and evaluated by autoradiography. (B) Western blot analysis of nuclear extract (N) and cytoplasmic fraction (C) prepared from MCF-7 cells. Samples were probed with antibodies against nuclear Rb protein and cytoplasmic Raf-1 protein. Sizes are given on the left in kilodaltons.

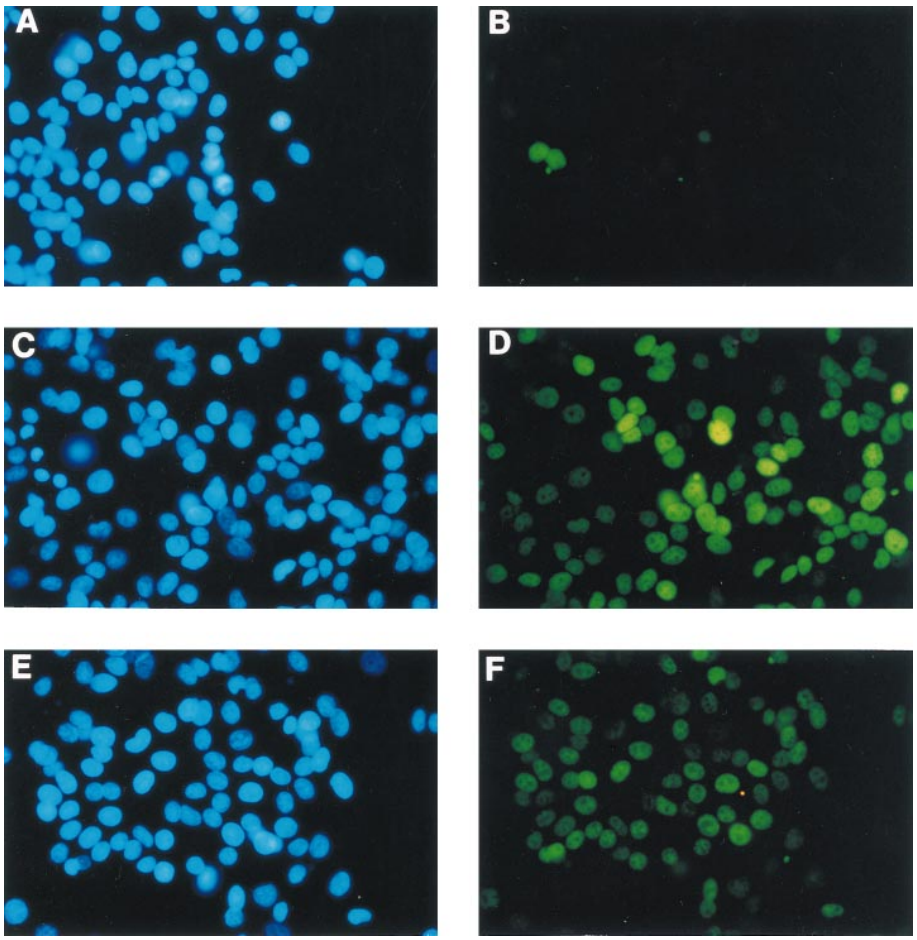

FIG. 6. Levels of p53 protein in untreated (A and B), actinomycin D-treated (C and D), and calpain inhibitor I-treated (E and F) MCF-7 cells. Cells were treated for 16 h with actinomycin D (5 nM) or 3 h with calpain inhibitor I (16 μM) as indicated, fixed, and probed for p53 protein with antibody DO-1 and fluorescein isothiocyanate-conjugated secondary antibody (B, D, and F). (A, C, and E) DNA was stained with DAPI.

while the p53 levels rose rapidly in RKO cells, barely any stabilization of p53 in the E6-expressing cells could be detected. This is in contrast to the results obtained with efficient proteasome inhibitors, where stabilization of p53 can be clearly detected in both E6-expressing and -nonexpressing cells (42). Degradation of p53 by E6 in vitro is inhibited by ATPγS, which inhibits degradation of ubiquitinated p53 through the proteasomes and results in the accumulation of polyubiquitinated p53 (Fig. 7B). Calpain inhibitor I had no effect on E6-mediated degradation of p53 in these in vitro assays (Fig. 7B), however, and no accumulation of ubiquitinated p53 was seen even at inhibitor concentrations more than 10-fold higher than those used to stabilize p53 in MCF-7 and RKO cells. Calpain inhibitor I was also shown to have no effect in the in vitro assay under conditions of reduced reticulocyte lysate (which provides the components for ubiquitin-dependent degradation) such that E6-dependent degradation was only weakly evident (data not shown). Taken together, these results indicate that the elevation of p53 levels in cells after treatment with calpain inhibitor I is by virtue of an inhibition of calpain rather than a direct inhibition of the ubiquitin-dependent proteolytic pathway. To verify that inhibition of calpain results in the stabilization of the p53 protein, we carried out pulse-chase experiments with untreated and calpain inhibitor I-treated RKO (Fig. 7C) and MCF-7 (Fig. 7D) cells. Calpain inhibitor I treatment clearly led to an increase of p53 half-life in both cell types.

DISCUSSION

Several mechanisms by which p53 activity may be regulated in cells, including modulation of conformation, phosphorylation, and protein stability, have been described. In this study we show that the p53 protein can be cleaved by calpain in vitro to generate an N-terminally truncated protein. Such a cleaved protein would retain many of the major functional regions of p53, including the oligomerization domain and the sequence-specific DNA binding domain, and could be predicted to retain some functions of the wild-type protein or show activity in the negative regulation the full-length protein. We have, however, been unable to clearly identify the cleavage product in cells, making it more difficult to assess the importance of the calpain cleavage in vivo. A p53 mutant which is resistant to calpain cleavage in vitro (p53ΔI) has been shown previously to be more stable when expressed in cells (37, 45), indicating a possible role for the cleavage in the regulation of p53 stability. In support of this suggestion, treatment of cells with calpain inhibitor I was shown to induce a rapid accumulation of p53 protein as a result of enhanced protein stability. This stabilization of p53 is not seen in E6-expressing cells, indicating that

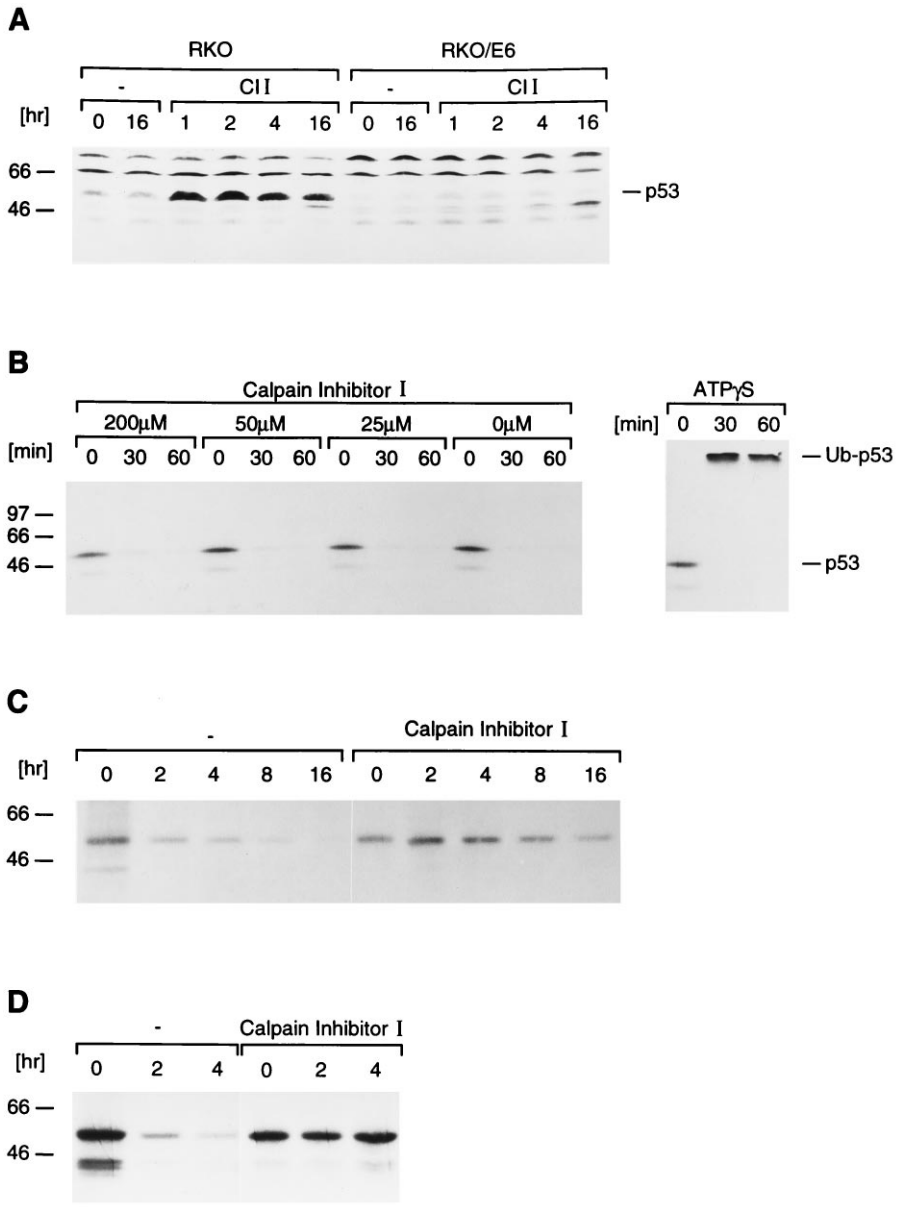

FIG. 7. (A) Western blot analysis of RKO and RKO/E6 cells after treatment with calpain inhibitor I (CI I). Cells were treated with calpain inhibitor I (16 μM) for the indicated times, and cell lysates were subjected to Western blot analysis using antibody PAb1801. (B) Effects of calpain inhibitor I and ATPγS on E6-mediated degradation of p53. In vitro-translated p53 was incubated with in vitro-translated E6 protein at room temperature in the presence of rabbit reticulocyte lysate and the indicated amount of calpain inhibitor I or ATPγS (2 mM). The position of the polyubiquitinated p53 (Ub-p53) is indicated. (C and D) Effect of calpain inhibitor I on the half-life of p53 in RKO (C) and MCF-7 (D) cells. Cells were preincubated with calpain inhibitor I (16 μM) for 1 h or left untreated (−). After 30 min of incubation in medium lacking methionine and cysteine, cells were labelled with medium containing 100 μCi of ^{35}S-labelled methionine and cysteine and then chased for different periods of time in medium supplemented with excess amounts of cold methionine and cysteine. p53 was immunoprecipitated with monoclonal antibody PAb421. Sizes are given on the left in kilodaltons.

at the concentration used the inhibitor is not significantly affecting proteasome function, consistent with previous studies showing that calpain inhibitor I is a much more potent inhibitor of calpain than of the proteasome (19, 51). Although calpain inhibitor II was somewhat less efficient in the stabilization of p53 in cells, requiring concentrations 5- to 10-fold higher than those of calpain inhibitor I to see an effect, this may reflect a difference in the relative efficiencies of these two inhibitors in vivo. These results are in agreement with a recent study analyzing the role of the proteasome in regulating p53 stability (42), where no stabilization of p53 was seen in cells treated with lower concentrations of calpain inhibitor II. Dissection of proteolytic activity resulting from proteasome or calpain function using inhibitors is complicated by the observation that in most cases both groups of proteases are affected, although their relative sensitivities differ.

Our inability to detect the p53 cleavage product seen in vitro in cells suggests that this smaller protein is not stable in vivo. It is possible that calpain itself mediates the further degradation of this protein, since we have noted that prolonged incubation with calpain in vitro results in further degradation of the cleavage product. In this case calpain may represent an alternative

pathway to the proteasome for the regulation of p53 stability. Another intriguing possibility is that the N-terminal cleavage of p53 by calpain is a prerequisite for recognition by the ubiquitin pathway and that mutant p53 proteins which are not cleaved by calpain, like p53ΔI, are resistant to both proteolytic pathways. Although factors governing recognition by the ubiquitin system are not well understood, there is evidence that residues at the N terminus of proteins play a role (3, 61), and it is possible that the new N terminus generated following calpain cleavage represents a more appropriate target for ubiquitin-dependent degradation than that found in the full-length protein.

The suggestion that calpain-dependent cleavage of p53 is necessary for subsequent ubiquitin-dependent degradation raises the interesting possibility that p53 stability following activation by DNA damage is regulated through calpain cleavage. Although we were unable to detect any reduction in the p53 cleavage activity in MCF-7 lysates prepared from DNA-damaged cells, in which the p53 can be shown to accumulate (data not shown), it is possible that DNA damage-induced modifications of the p53 protein inhibit recognition or cleavage by calpain. The localization of the preferred calpain recognition and cleavage site to N-terminal residues presents the potential for regulation of this activity by phosphorylation of residues within this region (48). Although mutation of the serine residue at amino acid position 15 failed to affect calpain cleavage in vitro, it is possible that phosphorylation protects the protein from cleavage and that the substitution of aspartatic acid in this position cannot adequately mimic phosphorylated serine. Interaction with other cell proteins may also modulate the stability of p53. The mdm2 protein, expression of which is regulated at the transcriptional level by p53, binds directly to the N terminus of the p53 protein and functions to inhibit transcriptional activity and some of the apoptotic functions of p53 (8, 26). The mdm2 binding site on p53 encompasses the region defined in this study as necessary for cleavage by calpain, and the p53ΔI mutant, which shows resistance to calpain cleavage, no longer interacts with mdm2. Recently, we found that expression of mdm2 leads to the degradation of wild-type p53 in vivo, although it has no effect on the stability of the ΔI mutant (27, 37). The mechanism by which this destabilization of p53 is achieved by mdm2 is not known, but it is interesting to speculate that the N-terminal cleavage of p53 by calpain is somehow involved in this process.

ACKNOWLEDGMENTS

We are extremely grateful to K. Cho (The John Hopkins University School of Medicine, Baltimore, Md.) for RKO and RKO/E6 cells, D. Morrison (ABL-BRP, Frederick, Md.) for the Raf antibody, and D. Lane (University of Dundee, Dundee, Scotland) for p53 antibodies.

This work was supported by the National Cancer Institute under contract with ABL.

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