Proteolysis by Calpains: a Possible Contribution to Degradation of p53

MAGALI PARIAT, SERGE CARILLO, MARTA MOLINARI, CATHERINE SALVAT, LAURENT DEBUSSCHE, LAURENT BRACCO, JO MILNER, AND MARC PIECHACZYK*

Institut de Génétique Moléculaire, UMR 9942, CNRS, 34033 Montpellier Cedex 01, 1 and Centre de Recherche, Rhône-Poulenc-Rorer, 94403 Vitry sur Seine Cedex, France, and Yorkshire Cancer Research Campaign p53 Group, Department of Biology, University of York, York YO1 5DD, United Kingdom 2

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p53 is a short-lived transcription factor that is frequently mutated in tumor cells. Work by several laboratories has already shown that the ubiquitin-proteasome pathway can largely account for p53 destruction, at least under specific experimental conditions. We report here that, in vitro, wild-type p53 is a sensitive substrate for milli- and microcalpain, which are abundant and ubiquitous cytoplasmic proteases. Degradation was dependent on p53 protein conformation. Mutants of p53 with altered tertiary structure displayed a wide range of susceptibility to calpains, some of them being largely resistant to degradation and others being more sensitive. This result suggests that the different mutants tested here adopt slightly different conformations to which calpains are sensitive but that cannot be discriminated by using monoclonal antibodies such as PAb1620 and PAb240. Inhibition of calpains by using the physiological inhibitor calpastatin leads to an elevation of p53 steady-state levels in cells expressing wild-type p53. Conversely, activation of calpains by calcium ionophore led to a reduction of p53 in mammalian cells, and the effect was blocked by cell-permeant calpain inhibitors. Cotransfection of p53-null cell lines with p53 and calpastatin expression vectors resulted in an increase in p53-dependent transcription activity. Taken together, these data support the idea that calpains may also contribute to the regulation of wild-type p53 protein levels in vivo.

The p53 protein is multifunctional and essential for maintenance of genomic integrity. It can function as a transcription factor (13, 46, 47, 53, 54, 76), but it has also been proposed to play a role in DNA repair (1), in homologous recombination (67), and in the regulation of the translation of its own mRNA (51). p53 can stimulate transcription of certain target genes in vitro and in vivo through the binding to specific DNA sequences termed PREs (p53-responsive elements) (3, 15, 18, 20, 29, 30, 56, 70) found in the promoter regions of genes involved in the control of the cell cycle, such as Waf1/Cip1 or the cyclin G gene (14, 16), and in DNA repair (52), such as GADD45 (28). It can also inhibit transcription of a variety of viral and cellular genes devoid of PREs, such as the human genes encoding c-Fos, β-actin (21), proliferating cell nuclear antigen (68), MDR1 (7), and basic fibroblast growth factor (69), probably by interacting with the TATA binding protein. Importantly, tumor-derived p53 mutants are no longer able to bind to PREs and to activate transcription (17, 30, 56, 70). Of paramount importance for its function, the p53 protein can adopt different tertiary structures that can be monitored by using a panel of monoclonal antibodies. Importantly, wild-type p53 protein can both promote and suppress cell proliferation, and these opposing functions correlate to three alternative conformational states of the protein (reviewed in references 46 and 47). Moreover, altered function of a number of mutant p53s found in tumors correlates with partially destabilized tertiary structure (47).

Posttranscriptional mechanisms play an important role in p53 gene regulation. In particular, p53 is one of the most unstable proteins found in higher eucaryotes. However, p53 can be dramatically stabilized in response to cues, such as DNA damage, that lead to half-life changes from minutes to hours (13, 23, 33, 34, 54, 61, 76), and a decrease in degradation rate is responsible for the high steady-state level of a number of mutant p53s found in spontaneously occurring tumors. A major route for p53 destruction has recently been shown to involve the ubiquitin-proteasome pathway (9, 38).

Calpains are a family of calcium-dependent intracellular proteases that can be divided into two major groups: ubiquitous calpains, termed milli- and microcalpains according to the concentration of calcium necessary for revealing their activity in vitro, and tissue-specific calpains (for reviews, see references 11 and 59). Ubiquitous calpains are stimulated by different proteins and phospholipids and are inhibited by a highly specific, high-molecular-weight protein inhibitor named calpastatin. Rather than peptide motifs, they recognize structural determinants, the nature of which remains to be characterized, and they usually cleave their substrates to only a limited extent. Ubiquitous calpains are usually considered exclusively cytoplasmic proteases. This observation is in accordance with the intracellular localization of most of their known or suspected substrates (also see Discussion). Using a series of in vitro degradation assays, we report here that wild-type p53 protein is a substrate for ubiquitous calpains and that degradation is dependent on p53 protein conformation. Modulation of calpain function in vivo, using selected cell lines, affected p53 steady-state levels and transcription functions. This finding indicates that activated calpains may also directly target p53 in living cells and that p53 breakdown may not occur exclusively by the ubiquitin-proteasome pathway.

MATERIALS AND METHODS

Chemicals. Bovine millicalpain (calcium-activated neutral protease), aprotinin, and soybean trypsin inhibitor were from Sigma, and leupeptin was from Boehringer Mannheim. Solid-phase synthesis of the calpastatin peptide inhibitor
(40) was performed on a Milligen 9050 peptide synthesizer, using the fluorenylmethoxycarbonyl group as temporary amino protection. AS1587, E64D, calpain inhibitor I, and calpain inhibitor II were from Sigma.

Cells, culture conditions, and preparation of cell extracts. Jurkat and SAOS cells were cultured in RPMI 1640 with 10% fetal calf serum supplemented with 2 mM L-glutamine and 50 mg/ml penicillin/streptomycin. They were harvested by trypsinization and verified by microscopic examination. Nuclei were removed by low-speed centrifugation (2,000 × g for 15 min), and supernatants were centrifuged at 100,000 × g for 1 h in a Beckman SW60 rotor. Cytoplasmic extracts (S100; 5 to 12 mg of protein per ml) were then aliquoted and frozen at −80°C until used (4). The rat, mouse, and human cells were described in reference 75. For translation in the presence of pure bovine microcalpain, the latter was usually added at a concentration of 10 μg/ml in the presence or absence of either E64D, calpain inhibitor I, or calpain inhibitor II at a concentration of 50 μM for the indicated period of times. Cytoplasmic extracts for immunoblotting analyses were prepared and processed as described elsewhere (48, 75).

In vivo and in vitro expression plasmids. For the translation of the human c-Fos protein and of hamster glyceraldehyde-3-phosphate dehydrogenase, we used plasmids pCDNAI-neo and pCDNA3 (4). Seven of the wild-type and site-directed mutant expression plasmids are described in reference 58. Others were obtained by site-directed mutagenesis of a wild-type p53 cDNA by using standard methods. For cell transfection experiments, we used the following plasmids: (i) RE-CAT, in which an HINdIII site of a human p53 cDNA was inserted downstream of the transcriptional control of the CON consensus sequence for p53 (36); (ii) NE-CAT, in which a HINdIII site of a human p53 cDNA was placed under the transcriptional control of the SV40 virus 40 late promoter and of the cytomegalovirus promoter, respectively. Absence of SV40 or reduced activity when compared for by addition of SV2ol and pCDNAI-neo, respectively, to keep constant the concentrations of both promoters in all transfection assays. MCF7 cells, seeded at a density of 105 cells per 60-mm-diameter culture dish, were transfected with various amounts of PM194, using Lipofectamine (Gibco/BRL) as specified by the supplier. The concentration of DNA was adjusted to 10 μg per transfection by using pCDNAI-neo. Immunoblotting analysis was carried out 36 h later.

RESULTS

Wild-type human and mouse p53 proteins are substrates for calpains in vitro. The sensitivities of human and mouse p53 to calpains were tested by using a previously described in vitro assay (4, 5; also see Materials and Methods). Briefly, p53 was produced by translation in a rabbit reticulocyte lysate, which is devoid of any detectable calpain activity, and then mixed with an S100 human Jurkat or Daudi cell cytoplasmic extract, which contains the calpain activity, and finally degradation reactions were started by adding calcium for activating calpains. Alternatively, purified bovine millicalpain was directly added to the reticulocyte lysate in certain experiments. Translation reactions most often gave rise to a relatively complex pattern of peptides. The longest peptide corresponded to the full-length p53, and the shorter ones resulted from either internal initiation or premature termination. Importantly, the suppressor conformation of the in vitro-translated proteins was confirmed before each degradation experiment by immunoprecipitation using monoclonal antibodies PAb240 and PAb1620 as previously described (41).

Initial degradation experiments were carried out in the presence of 200 μM calcium, which allows the activation of both micro- and millicalpins, since the former requires 2 to 75 μM calcium for half-maximum activity and the latter requires 200 to 800 μM (11). Similar results were obtained whether the protein was of human or murine origin. p53 was stable in the reticulocyte lysate for at least 2 h. When both calcium and the S100 cytoplasmic extract were added, p53 decayed rapidly (Fig. 1). Proteolysis was reproducibly two- to fivefold slower than that of the c-Fos proto-oncoprotein, which was processed in parallel and which is one of the most susceptible substrates identified for calpains (4, 5) (Fig. 1). Degradation was specific, since no decay of hamster glyceraldehyde-3-phosphate dehydrogenase and mouse dihydrofolate reductase was detected in experiments conducted in parallel (Fig. 1, lower panels). Degradation was limited, since a number of proteolytic products accumulated in the assay, in keeping with the notion that calpains usually degrade their substrates to a limited extent (11). The proteolytic product pattern was very simple in the case of the mouse protein, with the accumulation of two major bands of 33 and 41 kDa (also see below), and was complex in the case of human p53. In the latter case, the complexity is partly due to the complex pattern of translation products in the reticulocyte lysate that complicates the analysis. Typical degradation kinetic are presented in Fig. 1.

As a next step, we verified that calpains, but not another, unidentified calcium-dependent protease(s), were responsible for the cleavage of p53. Calpain activity (with no discrimination between micro- and millicalpins) can be identified by probing with an anti-glyceraldehyde-3-phosphate dehydrogenase antibody for verifying that the same amount of protein was analyzed in each sample. Cell transfection experiments. H358a and SAOS cells were seeded at a density of 2 × 104 cells per well of six-well culture plates (Nunc). They were transfected 18 h later by using Lipofectin (Gibco/BRL) as specified by the supplier, and CAT assays were performed after a further 36 h of culture according to standard procedures (8), using a Packard Instant Imager Instrument for analysis of thin-layer chromatography plates. Five hundred nanograms of the pre-CAT reporter plasmid SV53 p53 expression plasmid, when present, and various amounts of PM194 calpastatin expression vector were used per assay. p53 and calpastatin genes were placed under the transcriptional control of the simian virus 40 late promoter and of the cytomegalovirus promoter, respectively. Absence of SV40 or reduced activity when compared for by addition of SV2ol and pCDNAI-neo, respectively, to keep constant the concentrations of both promoters in all transfection assays. MCF7 cells, seeded at a density of 105 cells per 60-mm-diameter culture dish, were transfected with various amounts of PM194, using Lipofectamine (Gibco/BRL) as specified by the supplier. The concentration of DNA was adjusted to 10 μg per transfection by using pCDNAI-neo. Immunoblotting analysis was carried out 36 h later. Immunoprecipitations and immunoblotting analysis. Antibodies and conditions for immunoprecipitation of mutant and wild-type p53 have been described previously (41). Immunoblotting experiments were conducted essentially as described elsewhere (48, 75). Fifty micrograms of cell extract was used in the case of MCF7 and ts20 cells, and the immunodetection of p53 was performed with monoclonal antibody X77 (36) and an enhanced chemiluminescence kit from DuPont. Efficiency of protein transfer onto a nitrocellulose membrane was checked by using Ponceau red staining, and when necessary, immunoblotts were
using a series of protease inhibitors (for a review, see reference 73). On one hand, calpains are inhibited by calcium chelators such as EGTA, by cysteine protease inhibitors such as leupeptin, and most specifically by the highly specific endogenous inhibitor calpastatin or by a 27-mer synthetic peptide derived from it (called hereafter calpastatin peptide) (39). Calpains are not inhibited by soybean trypsin inhibitor and the serine protease inhibitor aprotinin. Calcium-dependent degradation of p53 was insensitive to soybean trypsin inhibitor and aprotinin but totally inhibited by EGTA, leupeptin, and the calpastatin peptide (Fig. 2A), thus demonstrating the involvement of calpains. We next showed that both micro- and millicalpain can induce p53 cleavage in vitro. First, cleavage of both mouse and human p53 was obtained in the presence of 50 μM calcium only (that is, a condition under which millicalpain is not activated) (Fig. 2B). The degree of degradation of p53 was, however, less at 50 μM than at higher calcium concentrations. This is most likely due to exhaustion of microcalpain activity under the experimental conditions used and not to differences in cleavage specificity between micro- and millicalpain, since a p53 protein added after 30 min of incubation was not degraded upon further incubation (not shown). Second, addition of purified bovine millicalpain directly to the reticulocyte lysate in the presence of 1 mM calcium caused rapid p53 decay (Fig. 2C). Finally, we demonstrated that p53 is itself a substrate for calpains in S100 cytoplasmic extracts rather than for another protease activated (directly or indirectly) through proteolysis by calpains. To this end, we used the following two-step degradation assay as follows: (i) p53 was first incubated for 30 min in Jurkat S100 extract in the presence of calcium, i.e., a time sufficient for activation of a second possible protease and degradation of the input p53 (Fig. 3A, lane b); (ii) fresh p53 was then added to the reaction, and incubation was continued for another 30 min under conditions that are permissive (i.e., in the presence of calcium) or nonpermissive (i.e., in the presence of EGTA or of the calpastatin peptide) for calpains. In the presence of calcium alone, p53 decayed rapidly, indicating that the p53-specific protease is not exhausted after 30 min of incubation (Fig. 3A, lane c). In contrast, calcium chelation (Fig. 3A, lane d) or, more significantly, the calpastatin peptide (Fig. 3, lane e) blocked degradation, thus implicating calpains as responsible for p53 proteolysis in the assay.

The degradation of pure recombinant wild-type mouse and human p53 by purified bovine millicalpain in vitro in the presence of calcium provided conclusive evidence that p53 is a substrate for calpains. At a high calpain concentration, a 33-kDa fragment prominently accumulated in the case of the mouse p53, in agreement with the size of the shorter proteolytic product generated in S100 cytoplasmic extracts. A more complex pattern of bands was again observed in the case of the human protein (Fig. 3B). It is of note that this experiment indicates that there is no absolute dependence on any cytoplasmic cofactor for p53 degradation by calpains.

In vitro-translated mouse p53 usually yields a simpler proteolytic product pattern than the human protein, as only two major peptides (peptide 1 [41 kDa] and peptide 2 [33 kDa]) are observed, thus making it a better candidate for the mapping of calpain cleavage sites. The peptide fragments were mapped by immunoprecipitation using monoclonal antibodies PAb248
p53 is a substrate for calpains. (A) Two-step degradation assay. The top panel outlines the experimental protocol. (a) p53 was incubated for 30 min in the presence of 200 μM CaCl2 in Jurkat S100 cytoplasmic extract. (b) The reaction mixture was split into three aliquots, and the reaction was continued for another 30 min in the presence of CaCl2 (c), CaCl2 plus EGTA (1 mM) (d), or CaCl2 plus the calpastatin peptide (0.5 mg/ml) (e). The full-length p53 is indicated by an arrowhead to the left of each gel. (B) Degradation of recombinant human and mouse p53 by millicalpain. One microgram of either recombinant human or mouse p53 (→) was incubated in the presence of various concentrations of pure bovine millicalpain in the presence of 1 mM CaCl2 for 1 h. Endpoint degradations (+), corresponding to 50 μg of calpain per ml, are presented. (m.c.) indicates an autoproteolytic product of millicalpain which is generated during the experiment and which can be visualized in degradation assays conducted with only millicalpain (not shown). (C) Immunotyping of mouse proteolytic products. Mouse p53 (indicated by an arrowhead) was degraded to only a limited extent (lane DigC) in a Jurkat S100 extract in the presence of 200 μM CaCl2. Immunoprecipitations were performed as described elsewhere (41), using monoclonal antibodies PAb421, PAb248, and PAb246. DigC corresponds to a polyclonal antiserum directed against the whole p53 protein. Tenfold more protein was loaded on the electrophoresis gel in the case of control p53 and of DigC-immunoprecipitated p53. (amino terminus), PAb421 (carboxyl terminus), and PAb246 (central core domain). Both peptides were immunoreactive for PAb246, but none of them was positive for PAb248 and only peptide 1 reacted with PAb421 (Fig. 3C). This result indicates that calpains cleave on both sides of the p53 central core domain responsible for DNA binding (2, 55). Reactivity with PAb246 indicates that the central core domain (peptide 2) retained conformational integrity. Peptide 2 appeared later than peptide 1 in degradation kinetics experiments (Fig. 1 and 2), suggesting that the N and C termini are sequentially removed, with the N terminus being cleaved first.

p53 sensitivity to calpains is determined primarily by the tertiary structure and not by the oligomerization state. Monomers of p53 assemble into dimers and higher-molecular-weight oligomers with high affinity via the oligomerization domain residing at the C terminus of the protein. Human and mouse p53 expression vectors linearized at unique SspI and StuI restriction enzyme sites, respectively, were used to produce monomeric proteins truncated within the oligomerization domain (49), which were tested for their sensitivity to calpains (Fig. 4). Truncated monomeric mouse and human p53 displayed a sensitivity close to that of full-length proteins, thus showing that an intact quaternary structure is not a prerequisite for cleavage.

As calpains recognize structural, rather than sequence, motifs, it was important to test the possibility that the various conformations of p53 are unequally sensitive to calpains. First, we showed that heat-denatured (65°C for 5 min) human and murine p53 are resistant to cleavage by calpains (Fig. 5A). The possibility of insolubilization upon heating that would have rendered p53 resistant to calpains was ruled out since under the same conditions, p53 retained its sensitivity to trypsin (not shown). We then used p53 mutants known to be temperature sensitive for conformation to examine whether conformation affects susceptibility to calpain degradation. Wild-type and mutant conformations of p53 are recognized by monoclonal antibodies PAb1620 and PAb240, respectively. The mouse A135V (45) and human A138V (73) p53 mutants were less susceptible to calpains in the PAb1620/PAb2400 conformation than in the PAb1620+/PAb2400 conformation (Fig. 5B and C). The two mutant proteins, however, behave slightly differently in degradation kinetic experiments. The mouse A135V mutant can quantitatively adopt the PAb1620+/PAb2400 conformation when translated at 27°C and the PAb16200/PAb24000 conformation when translated at 39°C. In contrast, A138V is totally in the PAb1620+/PAb24000 conformation at 39°C but displays a half-half phenotype when translated at 27°C (not shown). For the sake of homogeneity in our degradation experiments, sensitivity to calpains was tested at 37°C. This was possible be-
cause no detectable amount of protein translated at 39°C undergoes any conformation change when shifted to 37°C, at least for the duration of the experiment (not shown), and the conformation change occurs much more slowly than degradation by calpains when proteins translated at 27°C are shifted to 37°C. Experiments presented in Fig. 5C show that A135V translated at 27°C displays a sensitivity to calpains in S100 Jurkat cell extract comparable to that of the wild-type mouse protein whereas it is a poor substrate for calpains in its PAb1620/PAb240− conformation. Similarly, A138V proved to be a poor substrate for calpains when translated at 39°C and a better one when translated at 27°C (Fig. 5B). Its apparent degradation rate, however, turned out to be lower than that of the wild-type protein when translated at 27°C, with 50% degradation occurring after 5 min of reaction, compared to less than 1 min for wild-type p53. This observation is consistent with (i) the mixed phenotype of A138V at 27°C and (ii) the idea that the PAb1620/PAb240+ conformation of this protein is a poor substrate for calpains. Comparable observations were made when pure bovine millicalpain was added to in vitro translation mixes (not shown).

We next determined whether the p53 protein synthesized in vivo was also sensitive to calpains. For this purpose, we used the mouse T3T3 and 3T3tx cell lines, which are derived from BALB/c fibroblasts. Both lines express high amounts of p53 detectable by immunoblotting (48, 75). T3T3 p53 protein harbors two point mutations and adopts a mutant conformation (PAb240−) when produced in vitro in reticulocyte lysate. Surprisingly, it adopts a wild-type conformation (PAb1620+) when expressed in vivo. In this case, however, it is predominantly monomeric, with a small proportion being associated with a low-molecular-weight protein (48). 3T3tx p53 also carries two point mutations but is quantitatively found in high-molecular-weight complexes in vivo and displays a mutant conformation (PAb240−) (48). Cytosolic extracts from both cell lines were prepared, and the sensitivities to calpains of both p53 proteins were tested in parallel experiments (Fig. 6). Addition of calcium triggers the cleavage of T3T3 p53 and the subsequent appearance of two peptides with molecular weights close to those of peptides 1 and 2 (see above). Consistent with a role for calpains, the cleavage is inhibited in the presence of calpastatin or of EGTA. Notably, only half of the protein is broken down, even when exogenous calpain is added to the reaction mixture. The reason for this is not clear, but one possible explanation is that interaction with cell proteins controlling the tertiary and/or the quaternary structure of T3T3 p53 may protect p53 from attack by calpains. 3T3tx p53 was resistant to proteolysis in the presence of calcium even when purified bovine millicalpain was added to the extract. This finding rules out the possibility that the absence of cleavage was due to rate-limiting amounts of calpains in this cell context and supports the notion that 3T3tx p53 protein is resistant to calpains because of its mutant conformation.

**Natural mutant p53 proteins display different sensitivities to cleavage by calpains.** We next compared the susceptibility to calpains of wild-type p53 with those of 10 human p53 mutant proteins found in various types of natural tumors (R175H, M237I, M246V, R248W, R273C, R273H, R273P, T256A, V272M, and D281Y) and of three mouse mutants (C138H, C138P, and H176C (58)). All of the mutants exhibit size fractionation profiles similar to that of wild-type human p53, with peaks equivalent to monomers, dimers, and high-molecular-weight structures, in line with the idea that mutant proteins...
TABLE 1. Proteolysis of wild-type and mutant p53 proteins by calpains

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<tr>
<th>Protein</th>
<th>Mutant</th>
<th>Sensitivity to calpains</th>
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<tr>
<td>Human</td>
<td>A135V</td>
<td>PS (39°C)</td>
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<tr>
<td></td>
<td>R175H</td>
<td>PS</td>
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<td></td>
<td>M237I</td>
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<td>D281Y</td>
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<td>Mouse</td>
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* Sensitivities to calpains of the human and mouse wild-type proteins were compared to those of p53 mutants in parallel experiments conducted as described for Fig. 7. The flexibility of most mutants has been reported elsewhere (57) (also see text). VS, S, and PS correspond to very sensitive, as sensitive as wild-type p53, and poorly sensitive. See the text and legends to Fig. 5 and 7 for more information.

Increased p53-dependent transcription in SAOS and H358a cells transfected with a calpastatin expression vector. Direct assessment of the effect of calpains on p53 turnover in vivo is difficult for several reasons. First, calpains are reputed to be largely, if not exclusively, localized in the cytoplasm. Thus, if p53 is a substrate for calpains in vivo, it should be vulnerable for a short period of time after synthesis since, in principle, it should be protected as soon as it enters the nucleus (also see Discussion). Second, the regulation of calpain activity in vivo is unclear, and no means for exclusive activation of these proteases has ever been described. Third, no cell-permeant protease inhibitor that is strictly specific for calpains is currently available (11, 59, 73). To test the possible involvement of calpains in the regulation of p53 abundance, we initially used an indirect assay based on monitoring p53-dependent transcription in transient transfection experiments. To this aim, the p53-null SAOS (40) and H358a (19) cell lines were transfected with combinations of the following plasmids: (i) a wild-type human p53 expression vector (SVp53) in a limited amount to allow further p53-dependent transcription activity upon p53 stabilization; (ii) a reporter plasmid (RE-CAT) in which transcription of the bacterial CAT gene is under the control of the CON consensus DNA binding motif for p53 (20); and (iii) an expression vector for calpastatin (PM194), the physiological inhibitor of calpains in vivo. No stimulation of the reporter plasmid transcription was observed in the absence of p53 with any amount of calpastatin-expression plasmid. However, co-transfection of increasing amounts of the calpastatin expression vector, together with a constant amount of the p53 expression plasmid, led to a dose-dependent stimulation of p53 transcription activity. This level increased to five- and seven-fold in the cases of H358a and SAOS cells, respectively, under the experimental conditions tested (Fig. 8).
tumor cells, in which wild-type p53 accumulates to easily detectable levels, were transfected with increasing amounts of the PM194 calpastatin expression vector. The anti-p53 monoclonal antibody X77 (36) was used since it also recognizes an unknown protein of 30 kDa which did not vary and was thus useful as an internal reference (u.p. [for unknown protein] in Fig. 9). The results showed a dose-dependent accumulation of p53 (Fig. 9A) with a maximum threefold stimulation under the conditions used.

Reduced accumulation of p53 in ts20 cells treated with calcium ionophore. ts20 cells are thermosensitive for the E1 ubiquitin-activating enzyme of the ubiquitin cycle (9). At the non-permissive temperature (39°C), p53 continuously accumulates over a period of at least 24 h (not shown), because its degradation is essentially ubiquitin dependent in these cells cultured under standard conditions (9). To test the possibility that calpain activation could lead to a reduction in p53 abundance, ts20 cells cultured at 32°C were transferred at 39°C for 12 h, i.e., a time at which ongoing p53 synthesis provides a situation for proteolytic attack by calpains in the cytoplasm. The calcium ionophore A23187, which activates calpains in vivo, was then added, and p53 abundance was subsequently monitored as a function of time by immunoblotting using monoclonal antibody X77. Whereas p53 abundance increased up to 300 to 400% over a 4-h period in the control experiment, only a 50% increase was detected in the presence of calcium ionophore (Fig. 9B).

In addition to activating calpains, calcium ionophore induces a number of other intracellular events that might account for the control of p53 abundance. We therefore repeated the temperature shift experiment, with and without ionophore, this time adding separately protease inhibitors known to penetrate living cells. The inhibitors were E64D, calpain inhibitor I (also called N-acetyl-L-leucinyl-L-leucinyl-L-norleucinal, LLnL, or MG101), and calpain inhibitor II (also called N-acetyl-L-leucinyl-L-leucinyl-methionyl or LLM). None is strictly selective for calpains. E64D and calpain inhibitor I also inhibit cysteine proteases such as lysosomal cathepsins, but not the proteasome, whereas calpain inhibitor II also inhibits lysosomal proteases and the proteasome. However, all three inhibit calpains, and it follows that a common effect is likely to involve calpain inhibition. In the presence of ionophore alone, p53 failed to accumulate following a temperature shift to 39°C (Fig. 9C). This result is consistent with those of the previous experiment (Fig. 9B). However, when cells were treated with ionophore plus protease inhibitors, cellular p53 protein increased to levels equal to or greater than those induced in the absence of ionophore (Fig. 9C). Since all three inhibitors had similar effects,
the increased level of p53 likely resulted from calpain inhibition.

**DISCUSSION**

**p53 conformation and cleavage by calpains.** Our results demonstrate that both human and mouse p53 can be cleaved by milli- and microcalpains in vitro, and cell studies strongly suggest that p53 may also be a substrate for calpains in vivo. While this report was in revision, very similar results were reported by Kubbutat and Vousden for the human protein (33). Cleavage is independent of quaternary structure. However, p53 protein conformation appears crucial for calpain recognition and/or cleavage. Consistent with the notion that calpains do not fully degrade their substrates (11, 59), discrete and stable proteolytic products accumulated in the degradation assays used here for both human and mouse p53. Species-specific patterns were, however, observed. The motifs recognized and cleaved by calpains in their substrate proteins have not yet been clearly identified. They are likely to be of a conformational nature with no strict dependence on specific amino acids at the level of scissile bonds, although amino acid preferences have been reported (11, 59). Thus, it is not clear whether the different degradation patterns are due to slight conformational differences between mouse and human p53 that influence recognition by calpains or whether they are due to amino acid variations at the cleavage sites per se. It is important to note that the proteolytic cleavage of p53 by calpains is completely distinct from the proteolytic cleavage induced following interaction of p53 with damaged DNA (50). In the latter case, the cleavage of p53 is observed in calcium-free conditions and also with purified p53, ruling out any involvement of calpains. Nonetheless, there are some similarities between the two cleavage products (of 40 and 35 kDa following interaction with damage DNA) and a conformationally intact central core domain, reactive with monoclonal antibody PAb246 (Fig. 3C and reference 50). Since some functions of p53 are activated by amino and carboxy truncations, proteolytic cleavage may represent one mechanism regulating the numerous functions of p53.

Because they are sensitive to conformational motifs, calpains can be used as topological probes in protein structure studies. Very interestingly, the different mutants of p53 tested in our survey displayed very different sensitivities to these proteases. Some were as sensitive as the wild-type p53. However, some, such as R248W, R273C, and R273H, were more sensitive, while others, such as the human R175H, M237I, and R273P mutants showed a reduced sensitivity, and another category, which includes the mouse A135V and the human A138V mutants translated at 39°C, showed very low sensitivity. Our data thus support the notion that the different p53 mutants do not adopt a unique conformation but rather adopt a variety of conformations that cannot be discriminated by immunotyping using antibodies PAb240 and PAb1620. It is potentially interesting that the highly sensitive mutants were also more extensively degraded than the wild-type protein in the assay used (Fig. 7A). Whether mutation-linked conformational changes create or expose new cleavage sites to calpains is, however, not clear, since (i) due to the complexity of the pattern of translation products in the case of the human protein, the analysis of degradation products is usually difficult, and (ii) using certain batches of S100 cell extract, we have sometimes observed rather extensive cleavage of wild-type p53 (e.g., Fig. 2). Using a different assay involving a recombinant protein, Bargonetti et al. (2) have shown that (i) p53 possesses multiple cleavage sites for thermolysin on either side of a protease-resistant central core domain of 27 kDa displaying DNA binding activity and (ii) in the presence of thermolysin, certain mutants, such as R248W and R273H, showed a proteolysis profile similar to that of the wild-type human protein, whereas the core domain was degraded in the case of the V143A and R175H mutants, presumably because of conformation changes induced by the mutations which render cryptic cleavage sites accessible to the protease. Taken with our own observations, this work shows that susceptibility to various proteases may be used for identifying novel variant conformations of mutant p53 that cannot be detected by classical immunotyping.

**Contribution of calpains to p53 breakdown in vivo?** The mechanisms responsible for wild-type and mutant p53 protein breakdown are poorly understood, and multiple catabolic pathways may exist. Moreover, the relative contributions of different pathways may vary according to the cellular context and/or the physiological conditions. The ubiquitin-proteasome system is responsible for the destruction of numerous intracellular proteins (for reviews, see references 10, 22, 27, 29, and 61). Some evidence is consistent with a role for this system in the degradation of wild-type p53 in vivo, since p53 destruction has been shown to require energy (24) and an active E1 ubiquitin-activating enzyme, at least in mouse embryonic fibroblasts (9), and inhibition of the proteasome leads to stabilization of p53 and accumulation of ubiquitinated forms of p53 in human fibroblasts (38). One indication that p53 can be degraded by different mechanisms in vivo is the fact that E6 proteins from human papillomavirus types 16 and 18 have been shown to accelerate p53 destruction and thereby to favor the development of tumors of the cervix (63). In this case, degradation unambiguously involves tagging of p53 by multiubiquitin chains, due to a cellular protein called E6-AP that interacts with p53 only in the presence of E6 (10, 61, 62, 65). However, whether this corresponds to acceleration of a physiological p53 ubiquitin-dependent degradation pathway or to a deviation toward a more efficient one is not yet clear. It must also be stressed that p53 may also constitute a heterogeneous population of molecules with respect to intracellular proteolytic systems. This is exemplified by the fact that DNA-bound, but not free, p53 is resistant to E6-mediated degradation (50). Interestingly, neither the presence of E6 nor that of oligonucleotides carrying the CON consensus p53 DNA binding site (20) facilitates or inhibits p53 proteolysis by calpains in our in vitro degradation assays (results not shown).

We have shown here that the inhibition of calpains in transient transfection experiments leads to a significant elevation of p53-dependent transcription in SAOS and H358a cells. Moreover, elevation of p53 abundance was observed in MCF7 cells transfected with a calpastatin expression vector, and calpain inhibitors allowed p53 accumulation in ts20 cells upon activation of calpains by calcium ionophore (Fig. 8). Since p53 is a sensitive substrate for calpains in vitro, these observations raise the interesting possibility that calpains can also contribute to p53 breakdown in vivo and thus support the notion that p53 may not exclusively be degraded by the ubiquitin-proteasome pathway. We cannot, however, formally exclude the possibility that calpains regulate p53 steady-state levels by indirect mechanisms and/or modulate p53 transcriptional activity by interfering with intracellular signal transduction cascades. It is of note that the two types of regulation may not be mutually exclusive. If p53 is indeed degraded by calpains in vivo, the major issues will be (i) to determine the relative contribution of calpain-mediated destruction of p53 in respect to that of other intracellular proteolytic systems, such as the ubiquitin-proteasome pathway, (ii) to characterize which proteolytic en-
zymes reduce the calpain-generated peptides to amino acids, since neither we (this work and data not shown) nor Kubbetut and Voussen (33) could detect them in vivo, and (iii) to determine to what extent resistance to calpains can contribute to increased accumulation of mutant proteins in tumor cells. Concerning the last point, our results suggest that not all p53 mutants would be involved, since only a few of them showed resistance to proteolytic attack by calpains and some of them were even more sensitive. Along this line, it is worth emphasizing that not all p53 mutants are stabilized to the same extent, some mutants even remaining as unstable as the wild-type protein.

It may appear paradoxical that breakdown of a protein reputed to be nuclear might (in part) be achieved by a protease reputed to be exclusively cytoplasmic. It is, however, worth pointing out that (i) p53 has been reported to be cytoplasmic or partially cytoplasmic in various cell contexts, (ii) the transport of nuclear proteins into the nucleus does not always occur immediately after synthesis, (iii) although the possible presence of calpains in the nucleus is controversial, a minor fraction of ubiquitinated calpains has been reported to be nuclear in established cell lines (35), (iv) micro- but not millicalpains has been shown to be capable of entering the nucleus upon elevation of calcium concentration in digitonin-permeabilized A431 cells (43), (v) several nuclear matrix proteins (42, 44), as well as several transcription factors, have also been shown to be highly susceptible substrates for calpains in vitro (4, 5, 66, 72), (vi) apparently, calpain-dependent degradation of p53 could be obtained in a nuclear fraction of MCF7 cells in vitro (33), (vii) inhibition of calpains in vivo leads to higher c-Fos and c-Jun transcription factor activity in transient transfection assays (reference 29 and our unpublished observations), and (viii) during mitosis, the nuclear envelope gets disrupted, thus allowing interactions between nuclear and cytoplasmic proteins. Taken together, these observations support the notion that nuclear proteins can be substrates for ubiquitous calpains. However, whether proteolysis occurs mainly before (for controlling the abundance of proteins available for transport into the nucleus or the turnover of proteins with blocked nuclear transport, for example) and/or after transport of substrates into the nucleus will have to be elucidated.

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The first two authors contributed equally to this work.

ADDITION IN PROOF


REFERENCES

33. Lane, R. D., D. M. Allan, and R. Mellgren.