On the involvement of calpains in the degradation of the tumor suppressor protein p53

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Abstract A crude fraction that contains ubiquitin–protein ligases contains also a proteolytic activity of ~100 kDa that cleaves p53 to several fragments. The protease does not require ATP and is inhibited in the crude extract by an endogenous ~250 kDa inhibitor. The proteinase can be inhibited by chelating the Ca^{2+} ions, by specific cysteine proteinase inhibitors and by peptide aldehyde derivatives that inhibit calpains. Purified calpain demonstrates an identical activity that can be inhibited by calpastatin, the specific protein inhibitor of the enzyme. Thus, it appears that the activity we have identified in the extract is catalyzed by calpain. The calpain in the extract degrades also N-myc, c-Fos and c-Jun, but not lysozyme. In crude extract, the calpain activity can be demonstrated only when the molar ratio of the calpain exceeds that of its native inhibitor. Recent experimental evidence implicates both the ubiquitin proteasome pathway and calpain in the degradation of the tumor suppressor, and it was proposed that the two pathways may play a role in targeting the protein under various conditions. The potential role of the two systems in this important metabolic process is discussed.

Key words: Calpain; p53; Ubiquitin; Proteolysis

1. Introduction

Accumulating evidence suggests that the ubiquitin–proteasome pathway plays a major role in the degradation of p53 and other transcriptional activators. Recent evidence suggests that these proteins are also degraded by calpains, at least under certain conditions. It is therefore important to determine the role of the two proteolytic systems in these central metabolic processes.

The ubiquitin pathway plays an important role in the degradation of many short-lived regulatory proteins. Degradation of a protein via the system involves two successive steps, conjugation of multiple molecules of ubiquitin to the target protein and degradation of the tagged molecule by the 26S proteasome (reviewed in [1] and [2]). In vivo, Gronostajski and colleagues demonstrated that the degradation of p53 in cells requires ATP, a characteristic that is a hallmark in the activity of the ubiquitin system [3]. Chowdary and colleagues have shown that inactivation of E1, the first enzyme in the ubiquitin pathway, results in accumulation of the tumor suppressor [4]. Maki and colleagues showed that in the presence of lactacystin, a specific inhibitor of the 20S proteasome, p53 stabilizes, a process that is accompanied by accumulation of the intermediates p53–ubiquitin adducts [5]. These findings demonstrate that p53 serves as a direct substrate of the ubiquitin system. In contrast, calpain inhibitor II that is a weak inhibitor of the proteasome, did not affect the fate of p53. It should be noted that in all these studies where the ubiquitin system was inhibited, researchers could not identify proteolytic intermediate products that could have suggested that the system plays a secondary role in a process where the initial cleavage/marking is carried out by a different system. In vitro, immunodepletion of E1 leads to inhibition of degradation of p53 [6]. Utilizing both intact cells and a cell-free system, Scheffner and colleagues found that HPV oncoprotein E6 transforms cells, at least partially, by targeting p53 for degradation via the ubiquitin system [7,8].

Calpains are calcium-dependent cysteine proteinases present in a variety of cells (reviewed in [9] and [10]). Some of the enzymes are distributed ubiquitously, while others are tissue-specific. Most of the current knowledge about the enzymological and biological properties of calpains applies to the ubiquitous isozymes μ-calpain and macrocalpain that differ in the optimal Ca^{2+} concentration in which they are active (μM vs. mM concentrations, respectively). They localize mainly in cytoplasm. All the calpains characterized are heterodimers consisting of a large and a small subunit. The large subunit has a catalytic and regulatory domains whereas the small subunit, that is common to both isozymes, carries another regulatory calcium binding domain. The nature of calpain-catalyzed proteolysis is not digestive: rather, it proceeds in a limited manner and results in several cleavages. All cells that contain calpains, contain also specific protein inhibitors of these enzymes, calpastatins. In many cells, the inhibitor concentration exceeds that of the enzyme and is sufficient to block all Ca^{2+}-dependent proteolysis in unfractionated extracts. Consequently, a major unresolved problem involves the identity the physiological substrates of these enzymes. It has been reported that following activation, calpains are translocated to the cell membrane and are sequestered from their inhibitors. Therefore, many potential substrates of calpains are membrane or membrane-associated proteins such as growth factor receptors, adhesion molecules, cytoskeletal and microtubule-associated proteins. In this context it is important to mention that mutations in the muscle specific calpain 3 enzyme lead to limb-girdle muscle dystrophy [11]. A recent study has implicated calpains in the degradation of p53 [12]. The researchers have shown that in certain cell extracts, calpain is

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Abbreviations: ALLM, *N*-acetyl-Leu-Leu-methioninal; ALLN, *N*-acetyl-Leu-Leu-norleucinal; CM, carboxymethyl; DEAE, diethylaminoethyl; E1, ubiquitin-activating enzyme; E2 or UBC, ubiquitin-carrier protein or ubiquitin-conjugating enzyme; E3, ubiquitin-protein ligase; HPV, human papillomavirus; MG115, carbobenzoxyl-Leu-Leu-norvalinal; STI, soybean trypsin inhibitor

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probably in excess over its protein inhibitor and the suppressor can be cleaved in unfractionated lysate. Such exceptional cases have been also described, for example, in rat erythrocytes [13] and during the maturation and fusion of myoblasts [14]. To demonstrate the involvement of the proteinase in p53 degradation, the researchers used also calpain inhibitors that inhibit, although less efficiently, the 20S proteasome. As for other potential protein substrates of calpains, Hirai and colleagues and Carillo and colleagues reported that calpains may be involved in the degradation of c-Fos and c-Jun in vitro [15,16]. Stancovski and colleagues reported however that c-Fos is targeted by the ubiquitin system both in vivo and in vitro [17]. Liu and colleagues reported that the transcription factor inhibitor IκBα can be targeted by calpains in vitro [18]. It should be noted that here also researchers have shown that the ubiquitin system is involved in the process both in vivo and in vitro [19,20]. Furthermore, in this case the specific ubiquitin system-targeting signal has been identified and mutations in this signal lead to stabilization of the protein.

2. Materials and methods

2.1. Materials

Hiload Superdex 200HR (16×600 mm) column was from Pharmacia. Materials for SDS-PAGE were from Bio-Rad. Centricons and Centripreps were from Amicon. Most fine chemicals and proteinase inhibitors were purchased from Sigma. MG115, a 20S proteasome and calpain inhibitor, was a kind gift from Drs. J. Adams and R. Stein, ProScript Inc., Cambridge, MA. Purified calpain and calpastatin were prepared from human erythrocytes as described [13,14] or purchased from Sigma. Radioactive materials were from DuPont NEN. All other Chemicals were of high analytical grade.

2.2. Methods

2.2.1. Preparation of the labeled substrates. cDNAs for p53, c-Fos, and human N-myc in pSP65 vector have been described elsewhere [6,17]. The pKH6 vector containing human c-Jun was a generous gift from Dr. Dirk Bohmann (EMBL, Heidelberg, Germany). ^{35}S-methionine-labeled substrates were generated in vitro in a coupled transcription–translation reaction in wheat germ extract (TNT, Promega) and partially purified as described [6,21]. ^{125}I-labeled lysozyme was prepared by the chloramine T method as described [22].

2.2.2. Partial purification of the p53-cleaving proteinase. Rabbit reticulocyte lysates were prepared and resolved by anion exchange chromatography on DEAE cellulose into unadsorbed material (fraction I) and high salt eluate (fraction II) as described [21,22]. Fraction II was fractionated by $(NH_4)_2SO_4$ into fraction IIA (0–38%) [22]. Two milliliters of fraction IIA (20 mg/ml) were loaded onto a HiLoad Superdex 200 HR (16×600 mm) gel filtration chromatography column, and the proteins were resolved in a buffer containing 20 mM Tris-HCl, pH 7.2, 150 mM NaCl, and 2 mM DTT (buffer A), using an FPLC system (Pharmacia). Fractions (2.4 ml) were collected, and salt was removed by repeated concentration–dilution cycles using Centricon 30 microconcentrators with buffer A without NaCl. Fractions were finally concentrated to ~100 μl.

2.2.3. Determination of the p53-cleaving proteinase activity. Degradation of ^{35}S-methionine-labeled substrates and ^{125}I-labeled lysozyme was performed in a reaction mixture containing in a final volume of 25 μl: partially purified proteinase (fraction 31 or pooled fractions 31 and 32 from the Superdex 200 column as described above and in the legend to Fig. 1), 40 mM Tris-HCl, pH 7.6, 1 mM DTT, 1 mM $CaCl_2$, and the labeled substrate. Mixtures were incubated for 30 min at 30°C. Following incubation, reaction mixtures were resolved via SDS-PAGE and proteins were visualized either following fluorography and exposure to film or by using a PhosphorImager (Fuji, Japan). When the activity of different inhibitors was studied, they were preincubated for 5 min at 30°C in the presence of the proteinase prior to the addition of the substrates.

2.2.4. Conjugation assay. E6-dependent conjugation of ubiquitin to ^{35}S-labeled p53 in the presence of purified E1, E2 and a partially purified preparation of E3 (aliquots from the resolved fraction IIA; see above) was monitored as described [17,21].

3. Results

3.1. Gel filtration chromatography of fraction IIA reveals a proteinase that cleaves p53

In our efforts to resolve E6-AP, the E3 enzyme that is involved in E6-dependent degradation of p53, from the E3 that is involved in degradation of p53 in the absence of the HPV oncoprotein, we fractionated via gel filtration chromatography crude rabbit reticulocyte fraction IIA that contains all the known species of E3 enzymes [21,23]. Aliquots from the column fractions were assayed for formation of ubiquitin–p53 conjugates in the presence of purified E1 and E2 enzymes. Surprisingly, in a molecular mass region of ~100 kDa, we noted a strong proteolytic activity that degraded p53 to a limited extent (Fig. 1, fractions 30–32). The activity does not require ATP as the conjugation assay was carried out in the presence of the non-cleavable analog, ATP-γ-S. To rule the possibility that the proteinase utilizes the available α–β high energy phosphate bond, we carried out the assay in the absence of ATP-γ-S. The proteinase can cleave p53 in an energy-independent manner (not shown). Obviously, the process does not require also the other two conjugating enzymes, E1 and E2, that were present in the assay presented in Fig. 1 (not shown), and appears to be carried out by a unique proteinase.

3.2. Identification of a specific inhibitor of the p53-cleaving proteinase

The absence of the proteolytic activity from the crude extract (Fig. 1) and whole lysate [6] lead us to test the hypothesis that the activity is masked by a specific inhibitor. As can be seen in Fig. 2, the proteinase activity in fraction 31 can be inhibited by adding crude fraction II that contains, most probably, the inhibitor. In order to identify the inhibitor more precisely, we examined the different column fractions in the presence of the active proteinase. As can be seen in Fig. 2, fractions 21–27 (a peak at fraction 25 with a MW of ~250 kDa) contain an inhibitor of the cleaving activity.

3.3. Characterization of the p53 proteinase

To further characterize the proteinase, we tested the effect of pH and a selected group of inhibitors. The cleaving enzyme is a neutral proteinase that has an optimal activity at pH 7.6. At acidic and basic region pHs, the enzyme is inactive (not shown). As can be seen in Fig. 3, the Ca^{2+} chelators EDTA and EGTA strongly inhibit the enzyme. In addition, the cysteine proteinase inhibitors, leupeptin and E64, and the -SH alkylating agent NEM, also inhibit the enzyme. The calpain inhibitors ALLN and ALLM, and the proteasome inhibitor MG115 that inhibits also calpains [24], inhibit the enzyme as well. In contrast, the serine proteinase inhibitors PMSF, TPCK, TLCK, and STI, the aspartic proteinase inhibitor pepstatin A, and the Fe^{2+} chelator *o*-phenanthroline, did not have any effect. From the inhibitors profile it appears that the p53-cleaving activity is calpain. Kinetic measurements showed that the proteolysis of p53 is rapid: in 2 min almost all the substrate has been cleaved to high molecular weight intermediates, whereas after 20 min the degradation was complete (not shown).

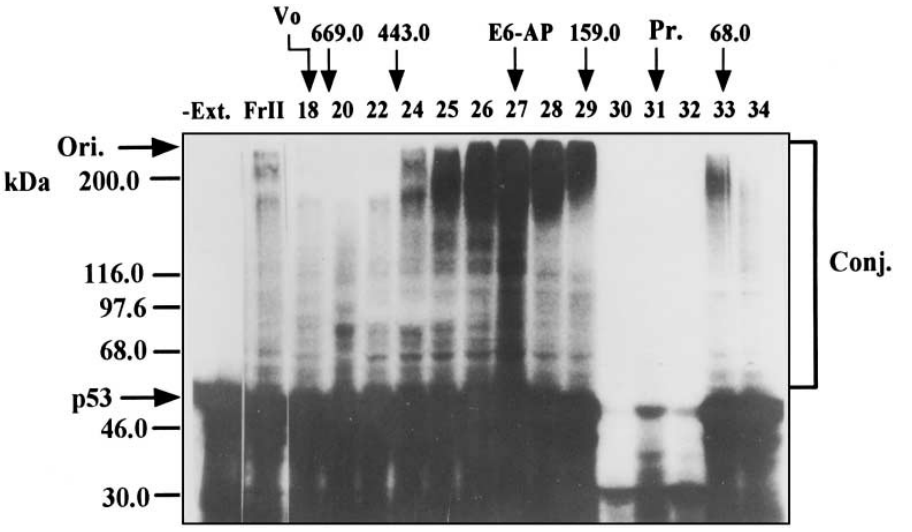

Fig. 1. Gel filtration chromatography of crude fraction IIA reveals a p53-cleaving activity. Ubiquitin–p53 conjugates were generated in the presence of E1, E2, the human papillomavirus oncoprotein E6 and 2 μl aliquots from the gel filtration-resolved fraction IIA as described under Section 2. Numbers denote fraction numbers. −Ext., reaction mixture incubated in the presence of all the components except for the ligase; FrII, a similar reaction incubated in the presence of crude fraction IIA prior to chromatography; V_o, void volume of the column; E6-AP, point of elution of E6-associated protein; Pr., p53-cleaving proteinase; p53, labeled substrate; Conj., p53–ubiquitin conjugates; Ori., origin. Gel filtration chromatography MW markers are: 669.0, thyroglobulin; 443.0, apoferritin; 159.0, aldolase; 68.0, bovine serum albumin. SDS-PAGE MW markers are: 200.0, myosin; 116.0, β-galactosidase; 97.6, phosphorylase b; 68.0, bovine serum albumin; 46.0, ovalbumin; 30.0, carbonic anhydrase.

3.4. The p53-cleaving enzyme is calpain

To test directly the notion that the p53-cleaving enzyme is calpain and that the inhibitor is calpastatin, we tested the effect of different calpain and calpastatin preparations on the proteolysis of p53. As can be seen in Fig. 4, all three preparations tested, the enzyme contained in fraction 31, a purified commercial calpain, and an homogeneously purified calpain from human erythrocytes, displayed an identical proteolytic profile. Furthermore, all three different inhibitor preparations, the one isolated from fraction IIA, commercially available calpastatin, and purified calpastatin from human erythrocytes, inhibit the three enzymes to the same extent. Thus, it is apparent that the p53-cleaving activity from fraction IIA is indeed calpain. It should be noted that similar to the activities in the crude extract, the molecular mass of calpain is 110 kDa whereas calpastatin is a multimeric inhibitor that appears frequently as a ~250 kDa complex [13].

3.5. Additional substrates of the p53-cleaving proteinase

To examine the substrate specificity of the calpain, we tested the activity in fraction 31 towards human N-myc, c-Fos, c-Jun and lysozyme. As can be seen in Fig. 5, myc, Fos and Jun were all proteolyzed by the enzyme. Calpastatin inhibits the degradation of all these proteins. Like p53, degradation of c-Fos yields intermediate products whereas the degradation of c-Jun and N-myc proceeds to completion. In-

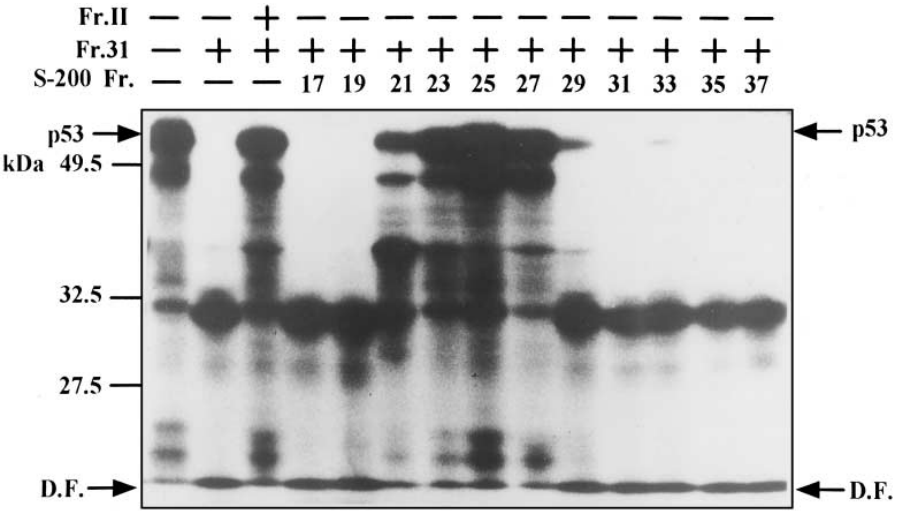

Fig. 2. Fraction IIA contains an inhibitor of the p53-cleaving activity. Aliquots (2 μl) of the different fractions (marked in numbers; see legend to Fig. 1) were assayed for the inhibitor of the p53-cleaving proteinase. Reaction mixtures contained also 2 μl aliquots of fraction 31 as a source of the proteinase. Proteolysis of ^{35}S-labeled p53 was monitored as described under Section 2. FrII, 1 μl aliquot of unresolved fraction IIA that was added as a source of the inhibitor. Molecular mass markers (stained) are: 49.5, ovalbumin; 32.5, carbonic anhydrse; 27.5, STI; D.F., density dye front. All other notes are as described in the legend to Fig. 1.

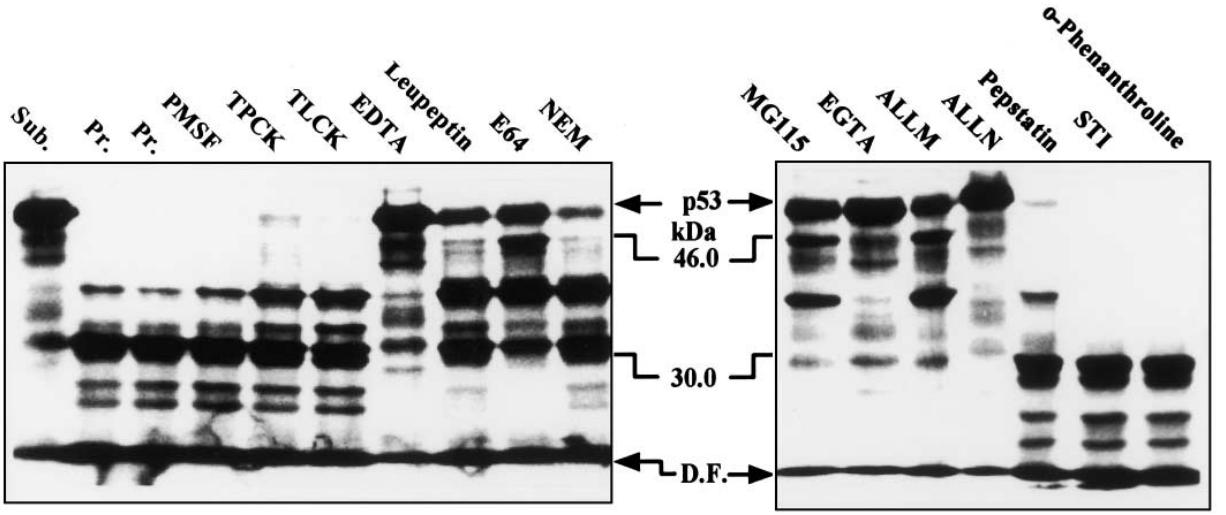

Fig. 3. Inhibitors of the p53-cleaving proteinase. Aliquots (2 μl) from pooled fractions 31 and 32 (used as a source of the proteinase) were assayed for p53-cleaving activity as described under Section 2 in the absence and presence of the indicated inhibitors. Sub., reaction mixture incubated in the presence of the labeled substrate alone; Pr., reaction mixture incubated in the presence of the proteinase. The concentration of the various proteinase inhibitors are: PMSF, 1 mM; TPCK, 1 mM; TLCK, 0.5 mM; EDTA, 2 mM; leupeptin, 1 μg/ml; E64, 10 μg/ml; NEM, 5 mM; MG115, 100 μM; EGTA, 2 mM; ALLM, 7 μg/ml; ALLN, 17 μg/ml; pepstatin, 1 μM; STI, 10 μg/ml; *o*-phenanthroline, 1 mM. Other notes and molecular mass markers are as in the legends to Figs. 1 and 2.

terestingly, lysozyme that is proteolyzed efficiently by the ubiquitin system, was not degraded by calpain. Thus, the calpain has a broad specificity, at least in vitro, and it is not specific to p53.

3.6. The ratio between calpain and calpastatin determines the activity of the enzyme in crude extracts

It appears that in most cases, the activity of calpains in crude extracts is masked by excess of calpastatin, and only dissociation and separation of the two leads to expression of the activity. One such exception is the rat erythrocyte in which a calpain-dependent cell fusion activity could be demonstrated following addition of the membrane mobility agent A_2C [13]. In contrast, such activity could not be demonstrated in human erythrocytes. Indeed, as can be seen in Fig. 6, crude rat erythrocyte fraction IIA cleaves p53, whereas human fraction IIA was inactive. The activity in the rat extract could be inhibited by calpastatin.

4. Discussion

We have shown that calpain can cleave p53 in a cell-free system, and that the proteolytic activity is inhibited by the endogenous native inhibitor of the enzyme, calpastatin. Since recent experimental evidence implicates both the ubiquitin system and calpains in the degradation of p53, we felt that it is important to review the data critically and to examine the possibility that the two systems cooperate in targeting the tumor suppressor protein for degradation.

In dissecting the potential role of the two systems, one can think of several distinct mechanisms. One obvious possibility is that calpains carry out the initial limited cleavages, whereas the ubiquitin system operates downstream and is involved in the terminal degradation of the intermediate products. Several strong experimental lines of evidence suggest that this order of events is unlikely. First, p53 is stabilized in the absence of energy without concomitant accumulation of intermediate products [3]. While both activation of ubiquitin and the activity of the 26S proteasome require energy in intact cells and in the cell-free system, activity of calpains appears to be energy-independent: calpains cleave proteins in crude extracts in the absence of ATP. It is still possible, although never been shown, that in the intact cell, energy is required also for calpain activity. For example, ATP may be required in order to translocate the enzyme to its site of action. Another experiment that suggests a role for the ubiquitin system in the initial

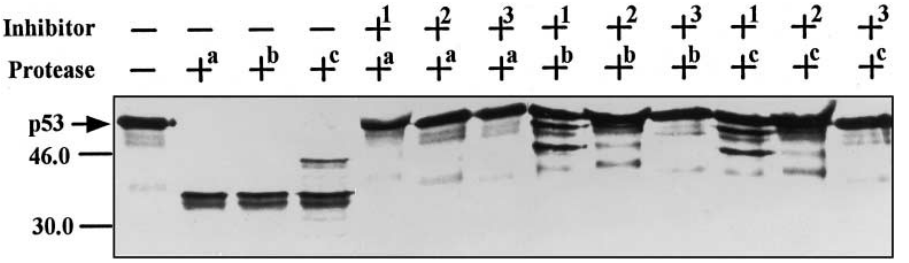

Fig. 4. The p53-cleaving proteinase is calpain. The partially purified proteinase[a] (Superdex 200 fraction 31; 2 μl aliquot), a commercial preparation of calcium-activated neutral proteinase[b] (0.05 U), and human erythrocytes purified calpain[c] (0.025 U; [13]) were assayed for p53-cleaving activity as described under Section 2. The fraction IIA-resolved inhibitor[1] (2 μl aliquot from fraction 25; see legend to Fig. 2), the commercial preparation of calpastatin[2] (0.15 U), and calpastatin purified from human erythrocytes[3] (0.075 U; [13]) were added as indicated. All other notes and molecular mass markers are as in the legend to Fig. 1.

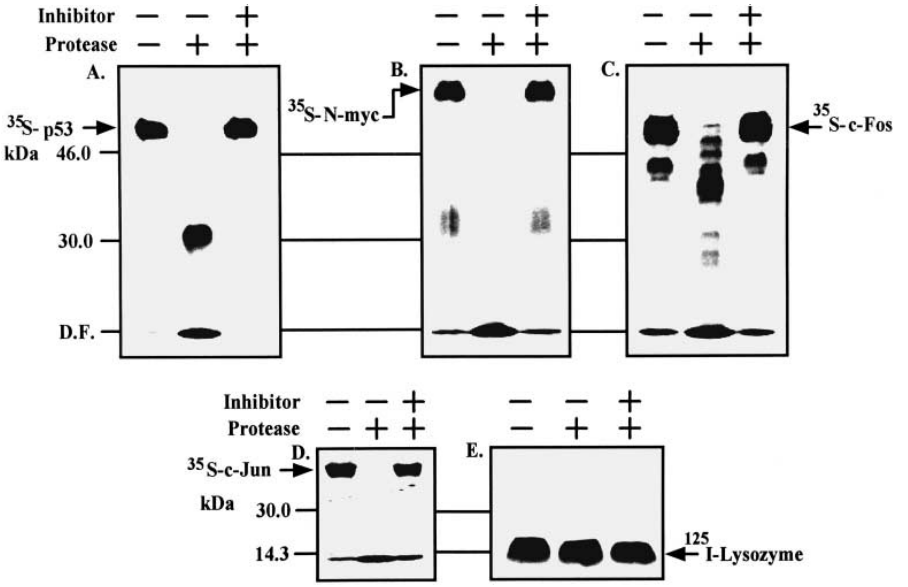

Fig. 5. Effect of calpain on proteolysis of human N-myc, c-Fos, c-Jun and lysozyme. Aliquot (2 μl) of fraction 31 were assayed for cleavage of p53 (A), human N-myc (B), c-Fos (C), c-Jun (D), and lysozyme (E) as described under Section 2. The inhibitor (2 μl, fraction 25, Fig. 2) was added when indicated. MW markers and notes are as described in the legends to Figs. 1 and 2.

proteolytic process and not downstream to calpains is stabilization of the suppressor following inactivation of E1, the first enzyme in the ubiquitin pathway cascade [4]. However, here too one may argue that E1 is not required directly for the ubiquitination of p53, but rather for activation of calpain in a yet unknown mechanism. However, there is no experimental evidence for such activation mechanism. Also, it should be noted that p53 has been shown to be a direct substrate of the ubiquitin system in vivo [5]. A second and distinct alternative is that the ubiquitin system is involved in the initial proteolytic event, whereas calpains act downstream. Here, the proteasome that acts as the proteolytic arm of the ubiquitin system is required for the initial cleavage event. Mechanistic dissection of the 20S proteasome reveals that the active sites in the β rings reside in a distance that allows, at the most, release of short peptides of ~8 amino acid residues [25]. Thus, the release of larger fragments will be difficult to explain. In one case however, processing of the p105 precursor of the NF-κB transcriptional activator, the proteasome is involved in limited proteolysis and not in complete degradation of its target protein substrate. In this exceptional case of a single and specific cleavage site, a glycine-rich region appears to inhibit translocation of the molecule into the proteasomal pore [26], and a single processing product is released. To further substantiate the downstream function of calpains it will be necessary to utilize specific inhibitors of these enzymes that do not inhibit the activity of the proteasome and to show accumulation of intermediate products that were generated by the proteasome. A third possibility is that p53 is targeted by different mechanisms in different cells. To demonstrate that the process is mediated by calpains, it will be necessary to show that the enzyme concentration exceeds that of its inhibitor or that the two components are localized to different cell compartments. In several cells and occasionally under different conditions, the molar ratio between the enzyme and the inhibitor changes and the enzyme can exist in its free form. For example, in maturing myoblasts, researchers noted a transient decrease in the level of calpastatin that leads, most probably, to activation of calpain and subsequent cell fusion that is an essential step in muscle development [14]. In the MCF-7 cell line where degradation of p53 could be demonstrated in the crude extract, calpain concentration probably also exceeds that of calpastatin [12], although it has not been shown directly. This is the case also in rat erythrocytes (see Section 3 and Fig. 6). To demonstrate a primary role for calpains in the degradation of p53 in these exceptional cases, it will be necessary to demonstrate that the process does not require energy and is not inhibited by specific proteasome inhibitors such as lactacystin. This has not been shown in a recent study in which calpains have been implicated in the basal, E6-independent degradation of p53 [12]. A fourth and distinct alter-

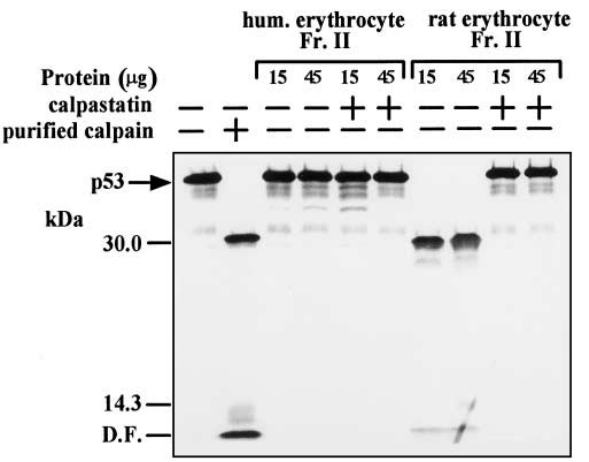

Fig. 6. Calpain is active in rat but not in human erythrocytes: rat and human erythrocytes were hemolysed and the membrane-free lysate (obtained by centrifugation of the lysate at 40000×g for 30 min) was resolved by anion exchange chromatography on DEAE-cellulose to fraction I and fraction II as described [22]. Labeled p53 was incubated in the presence of the indicated amounts of fraction II protein in the absence or presence of calpastatin purified from human erythrocytes (0.15 U; see legend to Fig. 4). Proteolysis p53 was monitored as described under Section 2.

native is that the two pathways are involved in different aspects of p53 degradation. For example, as has been recently suggested [12], calpain may be involved in regulating p53 degradation under basal conditions, whereas the accelerated E6-dependent process may be mediated by the ubiquitin pathway. It should be noted however that Maki and colleagues [5] reported that lactacystin, a highly specific inhibitor of the proteasome (that does not affect calpain activity), inhibits also the basal, E6-independent degradation of p53. This result along with the energy requirement for p53 degradation [3] and stabilization of the molecule following inactivation of E1 [4], processes that occur in cells that are not transformed by E6, leave the question of distinct pathways for E6-dependent and -independent degradation of p53 unresolved.

Interestingly, as can be seen in Fig. 5, some, but not all of the substrates of the ubiquitin system are also degraded by calpains. These include, in addition to p53, also N-myc, c-Fos, and c-Jun. In contrast, lysozyme, that is degraded by the ubiquitin system in vitro, is resistant to clapain. There are no obvious structural similarities between these substrates. In addition, while calpains are proteases, susceptibility to degradation by the ubiquitin system is determined to a large extent by the ability of the targeted proteins to be recognized by specific ubiquitin–protein ligases, E3s, and to be tagged by ubiquitin. Thus, it appears that the requirements for recognition by the two systems are clearly distinct and it is difficult to conclude whether the similarity (although as mentioned, not identity) in substrate profile between the two systems bears also a physiological relevance.

In conclusion, while one cannot rule out a role for calpains in regulating the stability of p53, it appears that a rigorous proof for their involvement in the process is still missing and that the ubiquitin system plays a major role in the process. Similar arguments can be raised also for the involvement of the two systems in the degradation of the other mentioned transcriptional regulators.

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