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Review Article

P53(ΔCp44), an Endogenous Human p53 Fragment Generated via M-Calpain-Mediated Cleavage Beyond Degradation

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Abstract

Endogenous fragments of p53 identified recently in human cytomegalovirus (HCMV)-infected human lung fibroblasts, specifically a ~44-kDa N-terminal fragment referred to as p53(ΔCp44), have been shown to be generated via m-calpain cleavage. p53(ΔCp44) appeared to be tightly associated with a chromatin-rich fraction, and was stabilized by the proteasome inhibitor MG132, particularly in mock-infected cells. The N- terminal p53 fragments were also present in three human dermal fibroblast cell lines tested, including fibroblasts isolated from post-burn hypertrophic scar. Understanding the biological functions of these fragments in the regulation of physiological and pathological processes, and the mechanisms regulating their generation and degradation, may shed light on currently unrecognized aspects of p53 regulation and function, and may provide a pathway for drug discovery.

Keywords: p53(ΔCp44); p53; calpain; cytomegalovirus

Abbreviations: HCMV: human cytomegalovirus.

Introduction

Regulation of the quantity and function of each protein inthe human body is pivotal in maintaining homeostasis. The amount of protein in a living cell or in the extracellular matrix depends on the balance between synthesis and degradation. Hence, intracellular and extracellular proteolysis also play an important role in maintaining functional protein concentrations. Insufficient or excess protein cleavage and degradation, particularly when combined with the dysregulation of biosynthesis, may lead to pathogenesis [1-3].

Peptidases or proteinases are now classified into seven families based on the nature of the catalytic residues [4-6] serine proteases [7] cysteine proteases [8] threonine proteases [9] aspartic proteases [10] glutamic proteases [11] metalloproteases [12] and asparagine peptide lyases [13]. It is crucial to degrade unnecessary or misfolded aberrant proteins and their aggregate proteins, as the abnormal proteins may be toxic to cells. The ubiquitin-proteasome pathway plays an important role in protein degradation [14,15]. In addition, some regulatory proteins are subject to rapid proteolytic degradation, which allows cells to rapidly adjust their concentration both temporally and spatially [16].Thus, it is also important to maintain the physiologically appropriate abundance of structural proteins. For example, matrix metalloproteinases (MMP) play an important role in modulating tissue turnover during fibrogenesis and cellular regeneration [17,18]. Insufficient extracellular protein fibrolysis or degradation may lead to excess fibrosis, as occurs in the development of keloids glond hypertrophic scars [18-20] and organ fibrosis [21-24].

The ubiquitin proteasome system and calpain are involved in the regulation of skeletal muscle catabolism, and an altered metabolic status may lead to a loss of lean body mass and muscle wasting [25-28].Some proteases are highly specific and only cleave substrates with a certain sequence through limited proteolysis, generating peptide fragments rather than destroying their substrates, and thus activating or inactivating the protein, or completely altering the protein's function [29-35]. The MEROPS database (http://merops.sanger.ac.uk) is an integrated source of information about peptidases, their substrates and inhibitors, which are of great relevance to biology, medicine and biotechnology [4-6].

Calpains May Modulate The Functions Of Their Substrates By Limited Proteolysis

Calpains are Ca^{2+}-activated non-lysosomal cysteine proteasesthat can cleave substrates in a limited fashion, besides completely degrading their target proteins [36-47]. Calpain-associated cleavage is essential to many calcium-regulated physiological processes, such as muscle contraction, neuronal excitability, secretion, signal transduction, cell cycle progression, cell proliferation, differentiation, apoptosis, and repair of damaged cell membranes [32,48,49]. Dysregulation of calpain is associated with multiple pathological processes, such as cardiovascular diseases, ischemic disorders, arterial sclerosis, muscular dystrophies, gastric ulcers, esophagitis, necrosis of activated hepatic stellate cells, fatty livers, pulmonary fibrosis, kidney diseases, neurodegenerative disorders, cataracts, vitreoretinopathy, diabetes, cancer, and infectious diseases [32,46,49-59].

Calpain-mediated limited cleavage can change protein function or potency, such that the protein acts significantly different from the parent protein. For example, the 18-kDa Bax fragment generated by calpain-mediated cleavage [60] displays a more potent ability to induce cell death than the 21-kDa full-length Bax [61] and the 17-kDa neurotoxic fragment of the tau protein generated by calpain-mediated cleavage may be a mechanism leading to neurodegeneration that is shared by multiple tauopathies [62,63]. Because specific amino acid residues or sequences have not beendefined for calpain-mediated proteolytic cleavage [44,46,64-67],calpain-mediated proteolysis may be associated with the conformation of the target proteins.

Endogenous Human P53 Fragments Generated Via M-Calpain-Mediated Limited Cleavage In Human Cytomegalovirus-Infected Cells

p53 tumor suppressor is a key regulatory protein, with essential functions as a transcription factor [68] and a translational regulator [69], participating in diverse cellular processes such as cell cyclearrest, DNA repair, apoptosis, and cell senescence [68,70-72] thatmodulate many physiological and pathological processes, including those in the digestive system [73-79]. Activities of p53, such as efficient and specific binding to p53 *cis*-elements within target promoter sequences, as well as tissue-, time-, and stimulus-specific binding of numerous coactivators and modifiers, are regulated by its abundance and post-translational modifications, which are influenced by a number of signaling pathways converging on p53 [68,80-88]. Constitutive synthesis and degradation maintain low levels of p53 in unstressed cells, but provide a mechanism for the rapid increase in cellular p53 levels in response to stress [89-91].

Human cytomegalovirus (HCMV) is a Δ herpesvirus that is responsible for serious infections in immunocompromised individuals, and in the developing fetus where it is associated with birth defects [92]. p53 is critical for HCMV infection [93-106]. Replication ofHCMV in quiescent host cells is dependent on activation of these cellsto enter and traverse the cell cycle to a point at or near the G_1/S boundary [107-109]. Paradoxically, contrary to the anticipated low quantities of p53 in cells entering the cell cycle, p53 quantities are substantially increased during productive HCMV infection [93,95,98,99,102] and remain at high levels for a protracted time during HCMV replication [102]. It has been shown that p53 is stabilized in HCMV-infected cells, which is partly associated with its resistance to proteasome-mediated degradation due to the break down and nuclear export of HDM2 [102] (Figure. 1). On the other hand, it has been known for some time that human p53 may be degraded by calpain (110-115), and that degradation of p53 by a calpain-like protease is necessary for G_1-to-S–phase transition [113]. Although the endogenous human p53 fragment generated via calpain-mediated cleavage was not reported earlier, it has been shown that exogenous p53 produced by *in vitro* translation in a rabbit reticulocyte lysate can be cleaved by m-calpain [112], generating some fragments.

Calpains are activated in HCMV-infected cells [116]. HCMV infection induces Ca^{2+} entry into infected cells [107], a substantial rise in intracellular free $[Ca^{2+}]$ [107] , which may activate the ubiquitous cellular calpains (Figure. 1). The activation of Δ- and m-calpain temporally overlap the increase in cellular p53 levels [116]. In HCMV-infected cells, at the times when calpain activities were apparent [116], high cellular levels of p53 were available without the potential confounding effects of rapid ubiquitin-facilitated p53 degradation [102] (Figure. 1). In fact, the cellular abundance and stability of p53 were greater in HCMV-infected cells than in mock-infected cells [93,95,98,99,102,117]. The changes in the sensitivity of p53 to calpain-mediated cleavage in HCMV-infected cells may also contribute to the resistance of most p53 molecules to degradation (Figure.1). The relationship of specific post-translational modifications of p53 to its sensitivity to degradation by m-calpain- mediated cleavage during HCMV infection to remains to be studied.

Although most p53 molecules are stable in HCMV-infected human lung fibroblasts, some p53 fragments, particularly p53(Δ Cp44), generated via m-calpain-mediated cleavage were identified recently [118]. That p53(Δ Cp44) is the product of m-calpain cleavage of p53 was demonstrated, for example, by the following approaches: [1] treatment of HCMV-infected cells with calpain inhibitors, E64d or ZLLH, either in the presence or absence of cycloheximide, substantially decreased the abundance of p53(Δ Cp44); (2) p53 extracted from either HCMV- ormock-infected cells was susceptible to cleavage by m-calpain *in vitro*, which generated p53(Δ Cp44), whereas Δ-calpain-mediated digestion did not produce additional p53(Δ Cp44) *in vitro*, although it degraded full- length p53. These and other results suggest that Δ-calpain is not responsible for generating many, if any, of the p53 fragments observed in HCMV-infected cells (Figure.1); [3] additionally, the susceptibility of p53 to m-calpain cleavage *in vitro* was enhanced when calpain-sensitive p53 molecules were preserved by pretreating cells with E64d, [4] and the increased levels of p53(Δ Cp44) in HCMV-infected cells were consistent with the activation of calpain in HCMV-, but not mock-, infected cells, as previously reported [116,118].

Calpain-Mediated Cleavage May also Collaboratewith other Protein Degradation Proteases

In our studies, the N-terminal p53 fragments generated via calpain-mediated cleavage may be further degraded via the ubiquitin pathway, as the proteasome inhibitor MG132 stabilized those fragments, including p53(ΔCp44), particularly in mock-infected cells [118]. Although greater quantities of p53(ΔCp44) were detected in HCMV-infected cells than that in mock-infected cells. These differences between in mock- and HCMV-infected cells may be due to the compromised ubiquitin-proteasome system in HCMV-infected cells [102]. The further degradation of these p53 fragments via the ubiquitin pathway suggests that calpain and ubiquitin systems may collaborate in the regulation of protein degradation (Figure . 1), especially when the latter pathway is not completely compromised by HCMV infection.
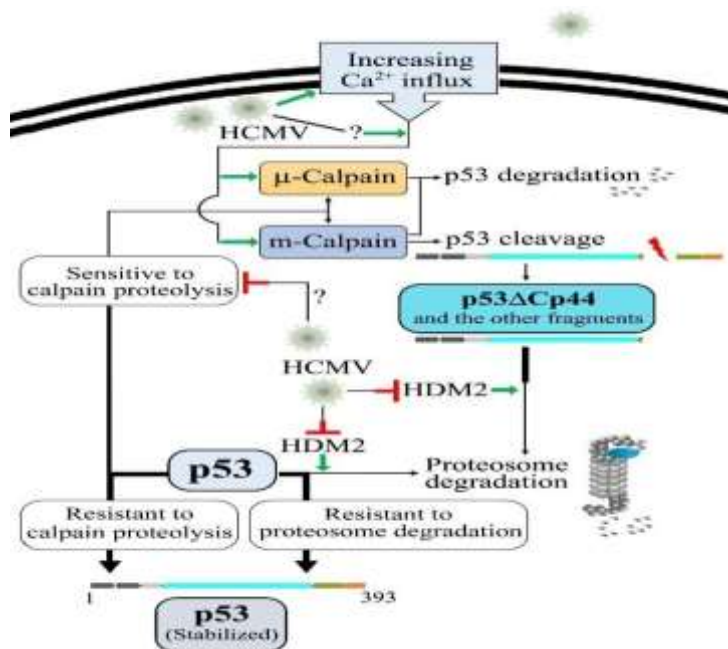
Figure 1. Sensitivity Vs Resistant of p53 to ubiquitin and calpain cleavage/degradation, and the generation of p53(ΔCp44) via m-calpain-mediated cleavage in HCMV-infected cells.

In HCMV-infected cells, most p53 are resistant to calpain-mediated proteolysis and proteasome-mediated degradation, although Δ- and m-calpains are activated, and m-calpains are able to cleave some p53 and generated some p53 fragments, including p53(ΔCp44). The p53 fragments can be further degraded via proteasome pathway, which is compromised in HCMV-infected cells, due to the decrease of HDM2. See text for detail.

The Biological Functions of p53 (∆cp44) and the other p53 n-Terminal Fragments Remain to be studied

Human p53 comprises 393 amino acid residues and six modular domains [68,86,88,119-121] as follow: [1] the N-terminus transcription activation domain contains two complementary transcriptional activation domains, with the major one at residues [1-42] and the minor one at residues [55-75]; [2] the proline-rich domain residues [61-92] ; [3] the central DNA-binding core domain residues [94-292]; [4] the oligomerization domain residues [326–353]; [5] the nuclear localization signaling domain residues [316-325] and [6] the C-terminal domain, which is involved in regulation of DNA binding, p53 protein stability, and transcription cofactor recruitment residues [364-393]. Among the p53 N-terminal fragments we observed by SDS-PAGE, fragments with a molecular mass of about 44-kDa, 47- kDa and 50-kDa could contain intact N-terminal structures, as they were detected with DO-1 and Bp53-12, since both antibodies recognize the N-terminal segment of p53 [122]. Although p53 appears to be a 53-kDa protein as determined by SDS-PAGE, size calculation based on amino acid residues yields a mass of only 43.7 kDa [123]. This difference may be due to the high number of proline residues in the proline-rich domain, which may slow p53 migration during SDS- PAGE and make it appear heavier than it actually is [123]. Because the proline-rich domain is located in residues [61-92], the N-terminal fragments observed should possess an intact proline-rich domain. Accordingly, based on the electrophoretic behavior of p53(ΔCp44) in SDS-polyacrylamide gels and considering the effect of the proline-richdomain, p53(ΔCp44) may lack approximately 70 amino acid residues at the C-terminus of p53. These missing residues contain most of the important domains, including the oligomerization domain, the nuclear localization signaling domain, and the whole C-terminal domain, thesemissing domains are subject to extensive post-translational modification, such as phosphorylation, acetylation, ubiquitination, sumoylation, methylation, and neddylation, and are critical for regulation of many biological functions controlled by p53 [68]. Nevertheless, the p53 protein has numerous other important active sites such as the transcription activation domain, the proline-rich domain, and the DNA-binding core domain, many of these sites will be preserved in p53(Δ Cp44). In fact, in our studies, p53(ΔCp44)appears to be predominately located in the nuclei of HCMV-infected cells and appears to be tightly associated with a chromatin-rich fraction. It is possible that one or more of the p53 N-terminal fragments binds to p53 response elements [124] and competes with thefunction of wild-type p53. Whether these p53 fragments bind to DNA indirectly by protein-protein interactions and/or directly via one or more of the domains remaining in the fragments has yet to be determined. Additional studies will be needed to define the precise mechanisms underlying the nuclear localization and tight chromatin-rich association of the p53 fragment identified here, as well as the possible effects of any p53(ΔCp44) binding.

N-terminal p53 fragments were also present in human dermal fibroblasts, including fibroblasts isolated from post-burn hypertrophic scar, hinting at a wider role for the p53(ΔCp44) fragment in other cellular systems [118]. p53 (ΔCp44) may also be part of a wider stress-associated, calpain-mediated response, making it worthy of future investigation.

Conclusion

Protein levels can be regulated at any of the steps in protein synthesis and degradation, from gene transcription, translation, post-translational modification including limited protein cleavage and complete breakdown. Great success has been achieved through small molecule drug discovery programs for the control of intracellular protein levels, particularly molecularly targeted therapy, and the new technologies are being developed [46,125-129]. The ubiquitin-proteasome system is important for degrading regulatory proteins and unnecessary, misfolded and/or aggregate proteins [14]. One novel approach uses Proteolysis Targeting Chimera (PROTAC) to degrade the functional target through the ubiquitin-proteasome system [129-133].

Calpains participate in the regulation of many physiological and pathological processes by performing either general or limited proteolysis, the latter of which does not destroy but rather may modulate the functions of these substrates. Therapeutic strategies targeting the activity of calpains have been developed to improve the specificity and bioavailability of calpain inhibitors [46,125]. Understanding the molecular mechanisms governing the regulation of calpain activity, the sensitivity or resistance of a target protein to calpain cleavage, the interplay and collaboration of calpain-mediated cleavage and the other protease systems, *e.g.*, the ubiquitin-proteasome system, and the function and regulation of new protein fragments generated by calpain-mediated cleavage, may shed light on novel pathways of new drug discovery.

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