Ubiquitin-Independent Degradation: Lessons from the p53 Model

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Abstract

Ubiquitin-proteasome degradation is a key cellular process involved in almost every aspect of cell life. According to the current concept, proteins are stable unless they are marked by poly-ubiquitination for degradation by the 26S proteasomes. A new twist in the concept became evident while studying the degradation of the tumor suppressor p53, a protein that appeared to satisfy this principle. We have discovered that native p53 is also prone to ubiquitin-independent 20S proteasomal degradation, suggesting that certain proteins are inherently unstable. We further found that this process of degradation is mediated by 20S proteasomes and inhibited by NADH quinone oxidoreductase 1. Our recent findings together with previous observations of ubiquitin-independent degradation suggest the existence of ubiquitin-independent mechanisms for proteasomal protein degradation in the cells.

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Protein degradation plays an important role in almost every basic cellular process, including cell cycle control, differentiation and development, cellular response to stress, DNA repair, transcription regulation, and regulation of the immune and inflammatory responses [1,2]. Degradation of most of the cellular proteins is carried out by the proteasomes [1,2]. The 26S proteasome is composed of a core 20S catalytic chamber, capped at both ends with 19S regulatory subunits. As discovered by the Nobel Prize laureates Avram Hershko, Aaron Ciechanover and Irwin Rose, the selective degradation of many short-lived proteins by the 26S proteasomes is mediated by the ubiquitin system. Ubiquitin, a 76 amino acid residue protein, is covalently conjugated in a highly regulated multi-step process to the substrate protein, marking it for degradation by the 26S proteasomes [1,2].

Many cellular proteins have been identified as substrates of the ubiquitin 26S proteasome degradation pathway. Among them are proteins involved in cell cycle regulation, transcription regulation, tumor suppressors, cell surface receptors and endoplasmic reticulum proteins, in addition to mutated and misfolded proteins [1,2]. The tumor suppressor p53 is one of the best-studied substrates of the ubiquitin-proteasome degradation pathway.

Ubiquitin-dependent degradation of p53

Wild-type p53 is a short-lived protein that accumulates following various types of stress and induces growth arrest or apoptosis. The level of p53 is tightly regulated by the rate of its proteasomal degradation [3]. Degradation of p53 via the ubiquitin-proteasome pathway was extensively studied. A major regulator of p53 stability is Mdm2, an E3 RING ubiquitin ligase that binds to the amino-terminal transactivation domain of p53 and ubiquitinates p53 [4,5]. p53 is further poly-ubiquitinated either by p300 or Mdm2 and the poly-ubiquitinated p53 is sensitized to 26S proteasomal degradation [6]. p53 is also ubiquitinated following its recruitment into a complex containing the viral E6 protein of the human papilloma viruses and the E6-associated protein, which serves as an HECT domain E3 ubiquitin ligase [7,8].

Recently, two additional E3 ubiquitin ligases – Pirh2 [9] and COP1 [10] – were demonstrated to poly-ubiquitinate p53 and induce p53 degradation. P*irh*2, a p53 target gene, encodes a RING-H2 domain-containing protein with ubiquitin-ligase activity [9]. P*irh*2 physically associates with p53 and promotes p53 ubiquitination independently of M*dm*2. Expression of P*irh*2 induces p53 degradation and represses p53-dependent transactivation and cell growth. *COP1* is another p53 target gene that encodes a RING domain E3 ubiquitin ligase [10]. *COP1* binds and ubiqitinates p53, targeting it for proteasomal degradation independently of M*dm*2 and P*irh*2. Degradation of p53 upon expression of *COP1* inhibits p53-dependent transcription and apoptosis. M*dm*2, P*irh*2 and *COP1* are all functionally similar, they are transcriptionally induced by p53, and they all encode RING domain-containing proteins that are capable of binding and ubiquitinating p53, targeting it for 26S proteasomal degradation. Thus, M*dm*2, P*irh*2 and *COP1* establish with p53 an autoregulatory negative feedback loop.

NQO1 and ubiquitin-independent degradation of p53

Recently we discovered that NAD(P)H quinone oxidoreductase 1, a ubiquitous enzyme that utilizes NAD(P)H to catalyze the reduction of various quinones, regulates p53 stability [11-17]. NQO1 binds and stabilizes p53 [15,17,18]. Binding of NQO1 to p53 is augmented in the presence of NADH and inhibited by dicoumarol, an inhibitor of NQO1, which competes with NADH [17]. Dicoumarol and several other inhibitors of NQO1 activity induce proteasomal degradation of p53 and inhibit p53-dependent apoptosis [11,14].

Several lines of evidence suggest that NQO1 regulates p53 stability via a unique pathway that is ubiquitin-independent and is mediated via the core 20S proteasomes. First, NQO1 fails to inhibit p53 degradation specifically mediated by the E3 ubiquitin ligase M*dm*2 [12]. Furthermore, a mutant p53 ($p53^{[22,23]}$) that is resistant to M*dm*2-mediated degradation is susceptible to dicoumarol-induced degradation [13]. Second, unlike M*dm*2-mediated degradation, dicoumarol-induced p53 degradation is not associated with accumulation of ubiquitin-conjugated p53 [13]. Third, inhibition of NQO1 by dicoumarol or NQO1 knockdown with specific NQO1 siRNA in cells with a temperature-sensitive E1 ubiquitin-activating

enzyme induces p53 degradation and inhibits apoptosis at the restrictive temperature under conditions devoid of ubiquitination [13]. Fourth, *in vitro* degradation studies showed that dicoumarol-induced p53 degradation is ubiquitin-independent [13]. I*n vitro* analysis with purified proteasomes further revealed that native p53 is degraded by the 20S, but not the 26S proteasomes. NQO1 together with NADH selectively protects p53 from degradation by the 20S proteasomes, and dicoumarol removes the protection [17]. Altogether, these findings establish a novel ubiquitin-independent mechanism for proteasomal degradation of p53 that is regulated by NQO1 and mediated via the 20S proteasomes in cells.

NQO1 regulates p53 stability in living cells under normal conditions. Experiments performed with cells that over-express NQO1 showed that under normal growth conditions these cells have an elevated level of p53 compared to the parental cells [12]. In accordance, knockdown of NQO1 with specific NQO1 siRNA reduces basal p53 levels [13]. Furthermore, NQO1 null mice exhibit reduced p53 protein levels and decreased apoptosis in the bone marrow [19].

NQO1 also determines p53 stability under different physiologic conditions. p53 accumulates following various types of stress, including γ-irradiation, oxidative stress and following exposure to different DNA-damaging agents [3]. Upon γ-irradiation p53 undergoes post-translational modifications and escapes M*dm*2-mediated degradation [3]. Interestingly, exposure to γ-irradiation also increases the binding of p53 to NQO1 [17]. Dicoumarol treatment abrogates p53-NQO1 interaction, inhibits p53 accumulation and blocks p53-dependent apoptosis following γ-irradiation [17]. Significantly, NQO1 knockdown with specific siRNA prevents p53 accumulation following exposure of the cells to γ-irradiation [17], indicating that NQO1 plays an important role in p53 accumulation following γ-irradiation. Escaping M*dm*2-mediated degradation is probably not sufficient for efficient p53 stabilization after γ-irradiation because p53 is still susceptible to 20S proteasomal degradation. Therefore, after γ-irradiation NQO1-p53 interaction is increased to eliminate p53 degradation by the 20S proteasomes.

The role of NQO1 in p53 accumulation after exposure to several DNA-damaging agents was demonstrated with NQO1 null mice. Studies performed with these mice revealed that treatment with benzo(a)pyrene fails to significantly increase p53 protein levels and apoptosis in the skin of NQO1 null mice compared to wild-type mice [20]. Therefore, these mice are more prone to develop benzo(a)pyrene and DMBA-induced skin cancer [20].

NQO1 might also play a role in p53 accumulation under oxidative stress. Accumulation of p53 following induction of oxidative stress by H_2O2 is more prominent in NQO1 over-expressing cells compared to parental cells [12]. NQO1 plays an important role in the cellular response to oxidative stress. Reactive oxygen species are known to induce expression of NQO1, which in turn reduces ROS [21]. The ability of NQO1 also to support p53 accumulation following oxidative stress may contribute to the cellular defense mechanism against ROS.

Although NQO1 regulates p53 stability via a distinct pathway that is both M*dm*2 and ubiquitin-independent, this pathway is also responsive to some of the regulators of M*dm*2-ubiqiuitin-dependent degradation. The tumor suppressor $p14^{ARF}$, which inhibits the ability of M*dm*2 to target p53 for degradation, also inhibits dicoumarol-induced p53 degradation [13]. Furthermore, the adenovirus E1A protein that stabilizes p53 by inducing $p14^{ARF}$ also inhibits dicoumarol-induced p53 degradation [13]. Thus, $p14^{ARF}$ has a double lock activity that inhibits p53 degradation by both the M*dm*2-dependent and the NQO1-regulated pathways, ensuring maximal p53 accumulation under certain physiologic conditions. Another interesting example is the viral oncogene SV40 LT that binds p53 and protects it from both M*dm*2-mediated degradation and dicoumarol-induced degradation [11,12].

The tumor suppressor p53 is mutated in more than 50% of human cancers [3]. Analysis of the susceptibility of wild-type and different p53 mutants revealed that the most frequent "hot spot" p53 mutants in human cancer, R175H, R248H and R273H, are resistant to dicoumarol-induced degradation [15]. These mutants remain sensitive to M*dm*2-ubiquitin-mediated proteasomal degradation [15]. Remarkably, these "hot spot" p53 mutants show increased binding to NQO1 compared to wt p53 [15]. Dicoumarol does not disrupt the binding of these "hot spot" p53 mutants to NQO1 and therefore fails to induce degradation of these mutants [22]. The ability of these p53 mutants to efficiently resist NQO1-regulated 20S proteasomal degradation may explain the relatively high steady-state expression levels of these mutants in the cells.

Ubiquitin-independent protein degradation

Traditionally it was thought that virtually all proteins that are degraded by the eukaryotic proteasomes must be ubiquitinated. However, several examples of ubiquitin-independent degradation by the proteasomes have been reported. The best-documented case is that of ornithine decarboxylase, the rate-limiting enzyme in polyamine biosynthesis. Accumulation of polyamines stimulates synthesis of antizyme, which binds to ODC and facilitates its degradation by the 26S proteasome without ubiquitination [23].

Several studies on the Cdk inhibitor $p21^{cip}$ suggested that the basal degradation of $p21^{cip}$ is ubiquitin-independent [24,25]. I*n vitro* studies have shown that $p21^{cip}$ binds to the α7 subunit of the 20S proteasome and is degraded by the 20S proteasomes in a ubiquitin-independent manner [26]. In contrast to the basal degradation of $p21^{cip}$, the degradation of $p21^{cip}$ following ultraviolet irradiation is ubiquitin-dependent [24]. Recent work suggested that proteasomal degradation of $p21^{cip}$ requires a functional ubiquitin system and that $p21^{cip}$ is ubiquitinated at the N-terminus [27]. The question of whether $p21^{cip}$ is degraded in a ubiquitin-dependent or ubiquitin-independent manner thus remains unresolved and calls for further investigation. It is possible that under different physiologic conditions $p21^{cip}$ is degraded via different mechanisms: ubiquitin-dependent or independent.

There are several additional examples for ubiquitin-independent degradation of proteins by the proteasomes. Degradation of the parvominute virus NS2 protein [28] and the degradation of retinoblastoma protein (p105) induced by cytomegalovirus pp71 [29] occurs without detectable polyubiquitination and under conditions that lack a functional ubiquitin-conjugating system in

cells harboring a thermo-labile E1 ubiquitin-activating enzyme. Experiments performed with c-Fos, an AP-1 transcription factor, have shown that its degradation in cells does not require poly-ubiqutination on internal lysines or N-terminal ubiquitination [30]. Furthermore, analysis of c-Fos degradation in cells harboring a thermo-labile E1 ubiquitin-activating enzyme suggests that both ubiquitin-dependent and ubiquitin-independent mechanisms are involved in c-Fos degradation [30]. I*n vitro* degradation of another AP-1 transcription factor, c-Jun, by the 26S proteasome does not require ubiquitination [31]. Additional experiments performed *in vitro* with purified 20S proteasomes showed that the microtubule-associated protein tau [32] and α-synuclein [33] are degraded by the 20S proteasomes without ubiquitination. However, it is still not clear whether the degradation of these proteins *in vivo* also occurs in a ubiquitin-independent manner.

Various oxidized proteins were reported as *in vitro* substrates of the 20S proteasomes, including oxidized glutamine synthetase, calmodulin, casein, superoxide dismutase, hemoglobin, myoglubin, albumin and oxidized histones [34]. Experiments in cells have shown that mild oxidation inactivates the ubiquitin-dependent system and the activity of the 26S proteasome but not of the 20S proteasome [35]. A study with cells harboring a thermo-labile E1 ubiquitin-activating enzyme demonstrated that these cells effectively degrade oxidized proteins in a ubiquitin-independent manner, further supporting the possibility that oxidized proteins are degraded by the 20S proteasomes *in vivo* [36].

Single protein, multiple degradation pathways

As outlined above, there is growing evidence that protein degradation can also occur by the proteasomes in a ubiquitin-independent manner. Previous studies on ODC degradation [23] and our recent findings on the ubiquitin-independent degradation of p53 and several other short-lived proteins such as p73 and ODC [17] suggest the existence of ubiquitin-independent mechanisms for proteasomal protein degradation in the cell. Ubiquitin-independent degradation can occur via the 26S proteasome, as evident in the case of antizyme-mediated ODC degradation [23], but can also be carried out by the 20S proteasomes, as in the case of the tumor suppressors p53 and p73 [17] and monomers of ODC [37,38].

In the past it was believed that a single protein is susceptible to a single distinct pathway of degradation. Nowadays it is becoming more and more evident that a single protein can undergo proteasomal degradation via different, distinct mechanisms, ubiquitin-dependent and independent, 20S or 26S proteasome-dependent [Figure 1]. Different E3 ubiquitin ligases (i.e., Mdm-2, COP1 and Pirh2) induce ubiquitin-dependent 26S proteasomal degradation of p53. p53 is also susceptible to ubiquitin-independent 20S proteasomal degradation that is regulated by NQO1 [17]. This is also the case with another p53 family member, the tumor suppressor p73α. Itch, an E3 ubiquitin ligase, can bind and ubiquitinate p73α, targeting it for degradation by the 26S proteasomes [39]. p73α is also degraded by the 20S proteasome in a ubiquitin-independent manner and is protected by NQO1 together with NADH [17]. Similarly, $p21^{cip}$ and c-fos are degraded

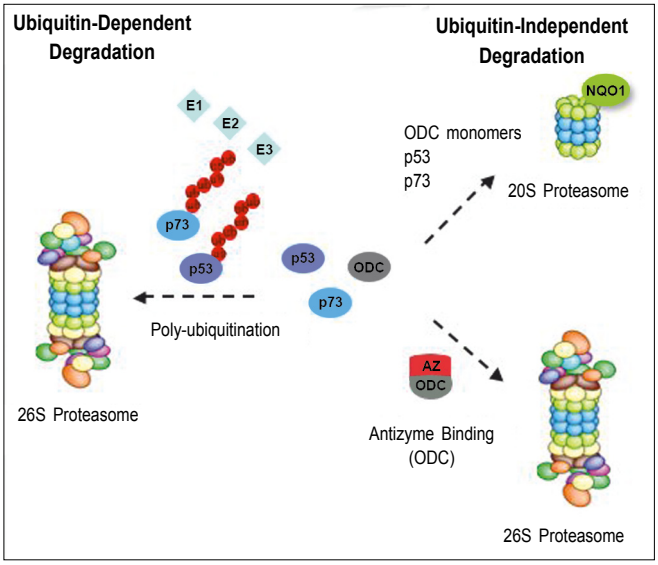

Figure 1. Schematic representation of ubiquitin-dependent and independent mechanisms for proteasomal protein degradation.

via both ubiqutin-dependent and ubiquitin-independent pathways. Finally, in addition to antizyme-induced degradation of ODC by the 26S proteasomes [23], ODC monomers are also susceptible to degradation by the 20S proteasomes unless protected by NQO1 [37,38]. In this exceptional case both the 26S and the 20S proteasomal degradation pathways are ubiquitin-independent. Furthermore, it seems that under different physiologic conditions a protein is susceptible to different pathways of degradation. This is nicely demonstrated with $p21^{cip}$, as basal degradation of $p21^{cip}$ is ubiquitin-independent whereas the degradation of $p21^{cip}$ following ultraviolet irradiation is ubiquitin-dependent [24,25]. It also seems that different mechanisms govern the degradation of a protein during different stages of its life. For example, newly synthesized proteins have a shorter half-life than old proteins [40], raising the possibility that these two different protein populations are susceptible to different mechanisms of degradation. It is possible that newly synthesized proteins have a shorter half-life than old proteins because they still did not acquire the correct folding and are therefore susceptible to rapid degradation by the 20S proteasomes.

Our findings that NQO1 is physically associated with the 20S proteasomes and that NQO1 can also bind and protect a subset of short-lived proteins from 20S proteasomal degradation suggest that NQO1 may function as a gatekeeper of the 20S proteasomes in the cells [17]. We propose a model whereby some short-lived proteins (such as p53, p73 and ODC) are inherently unstable and degraded "by default" by the 20S proteasomes in cells unless stabilized by a stabilizer. We have identified NQO1 as such a stabilizer. This degradation by default mechanism is distinct from the current "modification to destabilization" mechanism that is mediated by poly-ubiquitination. The nascent protein that manages to escape 20S proteasomal breakdown matures and is engaged in larger functional protein complexes. At this stage, the degradation of the protein can occur mostly via ubiquitin-dependent 26S proteasomal degradation.

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