Mdm-2 and ubiquitin-independent p53 proteasomal degradation regulated by NQO1

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The tumor suppressor p53 is a labile protein whose level is known to be regulated by the Mdm-2–ubiquitin–proteasome degradation pathway. We have found another pathway for p53 proteasomal degradation regulated by NAD(P)H quinone oxidoreductase 1 (NQO1). Inhibition of NQO1 activity by dicoumarol induces p53 and p73 proteasomal degradation. A mutant p53 (p53[22,23]), which is resistant to Mdm-2-mediated degradation, was susceptible to dicoumarol-induced degradation. This finding indicates that the NQO1-regulated proteasomal p53 degradation is Mdm-2-independent. The tumor suppressor p14ARF and the viral oncogenes SV40, p73 was detected with rabbit anti-human p73 antibody.

Materials and Methods

Cells, Cell Culture, and Compounds. The cell lines used were: HCT116 human colon carcinoma, p53 null HCT116, M1-t-p53 mouse leukemia, and COS 1 (described in refs. 15 and 20), 293 human kidney, A31N and A31N-ts20, a BALB/c mouse cell line that has a temperature-sensitive E1 ubiquitin-activating enzyme (21). A31N and A31N-ts20 cells were grown in DMEM (GIBCO) supplemented with 10% FBS, 100 units/ml penicillin, and 100 mg/ml streptomycin, and cultured at 32°C in a humidified incubator with 5.6% CO2. Dicoumarol, MG132, and thapsigargin were from Sigma, calpeptin was from Calbiochem, and tumor necrosis factor α was from PeproTech (Rocky Hill, NJ).

Transfection. Cells were seeded at 60% confluence in six-well plates 16 h before transient transfection with the desired plasmids. Transfections of 293 and HCT116 cells were performed by the calcium phosphate method. In HCT116, cells were treated with 10% glycerol for 30 s, 6 h after transfection. The amount of plasmid used in each experiment is indicated in the corresponding figure legend. Whenever needed, an empty vector was used to maintain a constant amount of total DNA in each transfection mixture. Transfections of A31N-ts20 BALB/c cells were performed with the lipofectamine reagent (Life Technologies, Grand Island, NY) according to the manufacturer’s protocol. Cell extracts were generally prepared 24 h after transfection.

Abbreviations: HA, hemagglutinin; NQO1, NAD(P)H:quinone oxidoreductase 1; siRNA, short interfering RNA; SV40, simian virus 40; LT, large T antigen.

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performed with a fluorescence-activated cell sorter, and the percent of sub-G₁ cells was determined.

**In Vitro Protein Degradation Assay.** Reticulocyte lysate degradation mixture contained: 40 mM Tris-HCl, pH 7.5, 5 mM MgCl₂, 2 mM DDT, 0.5 mM ATP, 10 mM phosphocreatine (Sigma), 1.6 mg/ml creatine phosphokinase (Roche Molecular Biochemicals), 200 µg/ml ubiquitin (Sigma), 50,000 cpm of in vitro reticulocyte lysate translated [³⁵S]methionine-labeled p53 (5% of the reaction volume), and the reticulocyte lysate fraction indicated. Degradation mixture was incubated at 37°C for 30 and 60 min and then mixed with Laemmli sample buffer, heated at 95°C for 5 min, and loaded on a SDS/12.5% polyacrylamide gel. After electrophoresis, gels were dried at 70°C for 2 h, exposed to x-ray film (Fuji), and developed. ATP dependence was determined by omitting ATP, phosphocreatine, and creatine phosphokinase and adding 20 mM 2-deoxyglucose and 20 µg/ml hexokinase to the reaction mixture. Ubiquitin dependence was determined by omitting ubiquitin from the reaction mixture and by using fraction II of reticulocyte lysate depleted of ubiquitin as described (24).

**Results**

**Inhibition of NQO1 Activity Induces Proteasomal Degradation of p53 and p73.** Inhibition of NQO1 activity by dicoumarol induces p53 proteasomal degradation (20). Dicoumarol also induces degradation of p73 (20) and this degradation was blocked by the proteasome inhibitor MG132 (Fig. 1A). Similar results were obtained by using the proteasome inhibitor lactacystin. Calcium-activated calpains can cause p53 degradation (25). However, elevation of intracellular Ca²⁺ by thapsigargin did not cause p53 degradation and the calpain-inhibitor calpeptin did not inhibit dicoumarol-induced p53 degradation (Fig. 1B), so that dicoumarol-induced p53 degradation is not mediated by calpain. Overexpression of wild-type NQO1, but not an inactive polymorphic NQO1, promotes p53 stability (15). In addition, transfection with a plasmid encoding NQO1-specific siRNA (Su-NQO1) reduced the level of p53, whereas the control RFX-specific siRNA (Su-RFX) did not (Fig. 1C). These results indicate a direct role of NQO1 in p53 and p73 stabilization and can explain the recent finding of reduced p53 and p73 levels in NQO1 knockout mice (26).

**Inhibition of NQO1 Activity Induces Mdm-2-Independent p53 Degradation That Is Blocked by p14ARF.** Ubiquitination is an important step in proteasomal protein degradation (18, 19). Mdm-2, an E3 ubiquitin ligase, ubiquitimates p53 and targets it to proteasomal degradation (9, 10), whereas SV40 LT stabilizes p53 (11). In contrast to p53, p73 is not targeted to degradation by Mdm-2 (27) and is not stabilized by LT (28). Unlike p53, dicoumarol-induced degradation of p73 was also not inhibited in COS-1 cells that overexpress LT (Fig. 2A). The p14ARF tumor suppressor, which inhibits the ability of Mdm-2 to target p53 for degradation (16, 17), also inhibited dicoumarol-induced p53 degradation (Fig. 2B). To study whether degradation of p53 by inhibition of NQO1 activity is mediated by Mdm-2, we used p53[22,23], a mutant p53 that cannot bind to Mdm-2 and is thus resistant to Mdm-2-mediated degradation (22). p53 null HCT116 cells were transfected either with wild-type p53 or p53[22,23] in combination with an Mdm-2-expressing plasmid and were then treated with dicoumarol. As expected, the degradation of wild-type p53 but not p53[22,23] was stimulated by Mdm-2 (Fig. 2C, lanes 1, 3, 4, and 6). However, both wild-type p53 and p53[22,23] were degraded in the presence of dicoumarol (Fig. 2C, lanes 2 and 5), indicating that dicoumarol induces p53 degradation in an Mdm-2-independent manner. The tumor suppressor p14ARF also protected p53[22,23] from dicoumarol-induced degradation (Fig. 2D, lanes 4–7).

**Inhibition of NQO1 Activity Induces Ubiquitin-Independent Degradation of p53.** Mdm-2-independent degradation of p53 can be induced by the human papilloma virus protein HPV-E6 (30),

![Fig. 1](image-url)
In all these cases proteasomal degradation of p53 is ubiquitin-dependent and is associated with p53 polyubiquitination. We therefore determined whether dicoumarol-induced p53 degradation is also associated with p53 polyubiquitination. Human kidney 293 cells were cotransfected with wild-type p53 alone or cotransfected with 3 μg of pCDNA3-HA-p14ARF. Twenty-four hours after transfection, cells were cultured for 5 h without (−) or with (+) 300 μM dicoumarol. (C) p53 null HCT116 cells were transfected with 150 ng of pRc/CMV human wild-type (wt) p53 or pRc/CMV human mutant p53[22,23] alone or cotransfected with 300 ng of pCDC-mdm2 X2. Twenty-four hours after transfection, cells were cultured for 5 h without (−) or with (+) 300 μM dicoumarol (Dic.). (D) p53 null HCT116 cells were transfected with 150 ng of pRc/CMV human mutant p53[22,23] alone or cotransfected with 3 μg of pCDNA3-HA-p14ARF or pCDNA3-E1A. Twenty-four hours after transfection, cells were cultured for 5 h without (−) or with (+) 300 μM dicoumarol (Dic.).

Fig. 2. Inhibition of NQO1 activity induces Mdm-2-independent degradation of p53 that is inhibited by LT, p14ARF, and E1A. (A) COS 1 cells expressing SV40 LT were γ-irradiated at 6 Gy and incubated for 4 h without (−) or with dicoumarol (Dic.). (B) p53 null HCT116 cells were transfected with 150 ng of pRc/CMV human wild-type p53 alone or cotransfected with 3 μg of pCDNA3-HA-p14ARF. Twenty-four hours after transfection, cells were cultured for 5 h without (−) or with (+) 300 μM dicoumarol. (C) p53 null HCT116 cells were transfected with 150 ng of pRc/CMV human wild-type (wt) p53 or pRc/CMV human mutant p53[22,23] alone or cotransfected with 3 μg of pCDNA3-HA-p14ARF or pCDNA3-E1A. Twenty-four hours after transfection, cells were cultured for 5 h without (−) or with (+) 300 μM dicoumarol (Dic.).

JNK (31), and adenovirus E4orf6 and E1B55K (32). In all these cases proteasomal degradation of p53 is ubiquitin-dependent and is associated with p53 polyubiquitination. We therefore determined whether dicoumarol-induced p53 degradation is also associated with p53 polyubiquitination. Human kidney 293 cells were cotransfected with wild-type p53 and HA-ubiquitin in the presence or absence of Mdm-2. The results show that transfection with Mdm-2 or treatment with dicoumarol induced a similar level of p53 degradation (Fig. 3, lanes 2 and 3). Whereas p53 degradation by Mdm-2 was associated with formation of high molecular weight p53-polyubiquitin conjugates, no such p53-polyubiquitin conjugates were detected after treatment with dicoumarol (Fig. 3, lanes 2 and 3, overexposed). This result indicates that dicoumarol-induced proteasomal degradation of p53 is not mediated by formation of polyubiquitin-p53 conjugates. To determine whether ubiquitin is required for dicoumarol-induced p53 degradation, we used a cell-free degradation system. Labeled p53 was added to a reticulocyte lysate-based degradation mixture supplemented with ATP, an ATP-regenerating system, and ubiquitin and incubated at 37°C for 90 and 180 min. As expected, p53 was stable under these conditions (Fig. 4A). However, p53 was efficiently degraded when either HPV-E6 (Fig. 4B) or dicoumarol (Fig. 4C) were added. When ATP and an ATP-regenerating system were omitted from the degradation mixture, dicoumarol-induced p53 degradation was not observed (Fig. 4D). This result indicates that dicoumarol-induced p53 degradation requires ATP, which is typical of a proteasome-mediated degradation system (18, 19). Similar experiments were performed by using a reticulocyte lysate degradation mixture depleted of ubiquitin (fraction II) (24). As expected, the HPV-E6-mediated ubiquitin-dependent degradation of p53 was completely prevented under these conditions (Fig. 4E), whereas dicoumarol still efficiently induced p53 degradation (Fig. 4F).

To examine further whether ubiquitin is required for dicoumarol-induced p53 degradation in cells, we used the A31N-ts20 BALB/c mouse cell line that has a temperature-sensitive E1 ubiquitin-activating enzyme (21). Only one E1 enzyme has been found in mammalian cells (33). Therefore, when these cells are cultured at the nonpermissive temperature (39°C), the E1 enzyme is inactivated and protein ubiquitination is repressed, facilitating accumulation of short-lived proteins such as p53 (21, 34). Addition of dicoumarol 24 h after cell transfer to 39°C induced p53 degradation (Fig. 5A, lanes 1 and 2). To rule out the
Inhibition of NQO1 Activity Induces Apoptosis in A31N-ts20 Cells at the Temperature Without Ubiquitination. Accumulation of p53 can lead to induction of apoptosis (5). We have previously shown that dicoumarol-induced p53 degradation in normal thymocytes and myeloid leukemic cells protects these cells against p53-mediated apoptosis (20). Culture of A31N-ts20 cells at 39°C for 48 h caused p53 accumulation and induced apoptosis (Fig. 5D and ref. 34). At this temperature, when ubiquitination is suppressed, dicoumarol induced p53 degradation (Fig. 5D, lanes 3 and 4) and completely inhibited apoptosis (Fig. 5D). These results indicate that the ability of dicoumarol to induce degradation of p53 can rescue cells from induction of apoptosis under conditions without ubiquitination.

Discussion

The tumor suppressor p53 is a short-lived protein that accumulates after different stress signals that decrease the ability of Mdm-2 to ubiquitinate p53. This accumulation can be achieved by posttranslational modifications of p53 and Mdm-2 or by their binding to other proteins (3, 4). By using an NQO1-specific inhibitor and overexpression of wild-type NQO1 or an inactive polymorphic NQO1, we have shown that NQO1 activity regulates p53 stability (15, 20). We now further show that reduction of endogenous NQO1 level by an NQO1-directed siRNA is sufficient to decrease p53 levels. p73 is also stabilized by NQO1 and our results can explain the recent demonstration of reduced p53 and p73 levels in NQO1 knockout mice (26).

DNA damage or oxidative stress can cause p53 stabilization because of inhibition of Mdm-2 activity. Overexpression of NQO1 showed even higher p53 stabilization under these conditions and did not inhibit p53 degradation induced by overexpression of Mdm-2 (15). These results suggested that p53 stabilization by NQO1 may not be mediated by inhibition of Mdm-2 activity. This possibility was now tested by using an Mdm-2-resistant mutant p53 (p53X22, 23). Our results indicate that induction of p53 degradation by inhibition of NQO1 activity is independent of Mdm-2. By using in vitro degradation assays and cells defective in ubiquitination, we show that this NQO1-regulated and Mdm-2-independent p53 degradation is also ubiquitin-independent. Thus, the NQO1-regulated p53 degradation pathway requires neither the E3-ubiquitin ligase Mdm-2 nor any E3 or other proteins involved in the ubiquitination pathway.

 Possibility that some residual E1 activity still remains in A31N-ts20 cells at 39°C, we tested for tumor necrosis factor-induced ubiquitin-dependent degradation of p53 without polyubiquitin-p53 conjugates. Human kidney 293 cells were cotransfected with 200 ng of pECoCMV human wild-type p53 and 1 μg of pEFRES-HA-ubiquitin (HA ubiq.) without or with 200 ng of pCOC-mdm2 X2. Twenty-four hours after transfection, cells were cultured for 5 h without (−) or with (+) 300 μM dicoumarol (Dic.).

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process. Our results show that p53 can undergo proteasomal degradation by two alternative pathways: one is ubiquitin-dependent and regulated by Mdm-2, whereas the other is ubiquitin-independent and regulated by NQO1. This finding implies that p53 stabilization is not solely dependent on inhibition of p53–Mdm-2 interaction but also requires NQO1 activity.

Both viral and cellular proteins can stabilize p53 by a protein-sequestration mechanism. The SV40 LT binds to p53 and prevents complex formation with Mdm-2 (11, 12), and the tumor suppressor p14ARF binds to Mdm-2 with the same outcome (16, 17). The adenovirus E1A oncogene stabilizes p53 by inducing p14ARF (29). Despite the lack of involvement of Mdm-2 in the NQO1-regulated p53 proteasomal degradation pathway, the ability of p14ARF to inhibit p53 degradation was maintained in this alternative pathway. Because p14ARF is known to function by interacting with Mdm-2, its role in the NQO1-regulated pathway was unexpected. However, at least under certain conditions, a direct interaction of wild-type p53 and p53[22,23] with p14ARF was observed (35), which may explain our data. Thus, p14ARF has a “double-lock” activity that inhibits p53 degradation by the Mdm-2-regulated and the NQO1-regulated pathways, which may ensure maximal p53 accumulation after different types of stress.

Cells accumulate p53 after exposure to oxidative stress, and NQO1 promotes p53 stabilization especially under oxidative stress (15). Accumulated p53 induces expression of the PIG3 (quinone oxidoreductase homolog) and FDXR genes and stabi-
lizes the p66Sc protein, which increases intracellular production of reactive oxygen species and promotes apoptosis of damaged cells (36, 37). Reactive oxygen species increase NQO1 activity (38), which in turn further stabilizes p53. This sequence of events is in agreement with the proposed feed-forward loop for p53 stabilization by reactive oxygen species (36).

Our finding that NQO1 stabilizes p53 and p73 has clinical implications. NQO1-deficient mice have reduced p53 and p73 levels and develop myeloid hyperplasia (26) and are more susceptible to development of carcinogen-induced cancer (39). Humans carrying the polymorphic C609T NQO1 gene that encodes a biologically inactive enzyme that fails to stabilize p53 (15) are more susceptible to tumor development (40, 41). The absence of a NQO1-regulated pathway for stabilization of tumor suppressors such as p53 and p73 can thus lead to tumor formation.

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