Regulation of p53 stability and p53-dependent apoptosis by NADH quinone oxidoreductase 1

Gad Asher, Joseph Lotem, Batya Cohen, Leo Sachs, and Yosef Shaul*

Department of Molecular Genetics, Weizmann Institute of Science, Rehovot 76100, Israel

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The tumor suppressor gene wild-type *p53* encodes a labile protein that accumulates in cells after different stress signals and can cause either growth arrest or apoptosis. One of the p53 target genes, p53-inducible gene 3 (*PIG3*), encodes a protein with significant homology to oxidoreductases, enzymes involved in cellular responses to oxidative stress and irradiation. This fact raised the possibility that cellular oxidation–reduction events controlled by such enzymes also may regulate the level of p53. Here we show that NADH quinone oxidoreductase 1 (NQO1) regulates p53 stability. The NQO1 inhibitor dicoumarol caused a reduction in the level of both endogenous and γ-irradiation-induced p53 in HCT116 human colon carcinoma cells. This reduction was prevented by the proteasome inhibitors MG132 and lactacystin, suggesting enhanced p53 degradation in the presence of dicoumarol. Dicoumarol-induced degradation of p53 also was prevented in the presence of simian virus 40 large T antigen, which is known to bind and to stabilize p53. Cells overexpressing NQO1 were resistant to dicoumarol, and this finding indicates the direct involvement of NQO1 in p53 stabilization. NQO1 inhibition induced p53 degradation and blocked wild-type p53-mediated apoptosis in γ-irradiated normal thymocytes and in M1 myeloid leukemic cells that overexpress wild-type *p53*. Dicoumarol also reduced the level of p53 in its mutant form in M1 cells. The results indicate that NQO1 plays an important role in regulating p53 functions by inhibiting its degradation.

The wild-type *p53* gene is a tumor suppressor gene that is often mutated in various tumors (reviewed in refs. 1 and 2). p53 accumulation and activation induces either growth arrest (1, 2) or apoptosis (3–6). p53 is a very labile protein, with a half-life as short as a few minutes (7). The rapid degradation of p53 is largely achieved through the ubiquitin proteasome pathway. The accumulation of p53 in response to DNA damage and other types of stress occurs mainly through posttranslational modifications. Proteins known to alter p53 stability include HPV16-E6 (8), simian virus 40 (SV40) large T antigen (9, 10), adenovirus E1B/E4orf6 (11), WT1 (12), and Mdm2 (13, 14). Whereas association of SV40 T antigen or WT1 with p53 increases p53 stability, the binding of E6 or Mdm2 to p53 accelerates its degradation (8–14). Many biological functions of p53 are attributed to its ability to function as a sequence specific transcriptional activator of selected genes (1, 2). One of the target genes of p53, p53-inducible gene 3 (*PIG3*), encodes a protein that shares significant homology with oxidoreductases from several species (15), raising the possibility of p53 regulation by oxidoreductases.

NADH quinone oxidoreductase 1 (NQO1) is a ubiquitous cytosolic flavoenzyme that catalyzes two-electron reduction of various quinones, with NADH or NADPH as an electron donor. This NQO1-mediated reduction mechanism is responsible for the cellular defense against various damaging quinones (16); however, some nontoxic quinones such as β lapachone are reduced by NQO1 to become toxic to cells (17). Expression of the *NQO1* gene is induced in response to a variety of agents, including oxidants, antioxidants, and ionizing radiation (reviewed in ref. 18), and *NQO1* expression is altered in a number of cancers, including breast, colon, and lung cancers (19–22). NQO1 is inhibited by dicoumarol [3,3′-methylene-bis(4-hydroxycoumarin)], which competes with NADH or NADPH for binding to the oxidized form of NQO1 and thus inhibits NQO1 activity (23).

We have now investigated the relationship between NQO1 and p53. Our results show that inhibition of NQO1 activity by dicoumarol caused enhanced p53 proteasomal degradation that can be prevented by overexpression of *NQO1*. The ability of NQO1 inhibition to enhance p53 degradation resulted in the reduction of p53 accumulation and suppression of p53-dependent apoptosis in γ-irradiated normal thymocytes and in M1 myeloid leukemic cells that overexpress *p53*. Our findings indicate the involvement of NQO1 in p53 regulation.

Materials and Methods

Cells and Cell Culture. The cell lines used were HCT116 human colon carcinoma cells, COS 1 monkey kidney cells, normal thymocytes obtained from 2.5-month-old BALB/c mice, and M1-t-p53 mouse myeloid leukemic cells that express a temperature-sensitive mutant p53 (Val-135) protein (3). The p53 in M1-t-p53 cells behaves like a tumor-suppressing wild-type p53 at 32°C and like a mutant p53 at 37°C (24). HCT116 and COS 1 cells were grown in DMEM supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 mg/ml streptomycin and cultured at 37°C in a humidified incubator with 5.6% CO_2. Normal thymocytes and M1-t-p53 cells were grown in DMEM supplemented with 10% heat-inactivated (56°C, 30 min) horse serum and cultured at 37°C in an incubator with 10% CO_2.

Compounds. Dicoumarol (Sigma) was dissolved in 0.13 N NaOH, and MG132 and lactacystin (Sigma) were dissolved in DMSO.

Establishment of Hemagglutinin (HA)-NQO1 Overexpressing Cell Lines. The coding region of human *NQO1* (GenBank accession no. J03934), including a 5′ influenza HA tag, was inserted into the pEFIRES expression vector containing a puromycin resistance gene (25). HCT116 cells (2.5×10^6 cells, in 10-cm plates) were transfected with 10 μg of purified pEFIRES-HA-NQO1 plasmid with the use of the Superfect transfection reagent (Qiagen, Chatsworth, CA). Puromycin-resistant colonies expressing HA-NQO1 were identified by immunoblot analysis with the use of anti-HA antibody.

Immunoblot Analysis. Cell extracts were prepared by lysis of PBS-washed cells in RIPA lysis buffer [150 mM NaCl/1% Nonidet P-40 (vol/vol)/0.5% AB-deoxycholate (vol/vol)/0.1% SDS (vol/vol)/50 mM Tris·HCl (pH 8)/1 mM DTT/1 μg/ml each of leupeptin, aprotinin, and pepstatin (Sigma mixture)]. The

Abbreviations: HA, hemagglutinin; NQO1, NADH quinone oxidoreductase 1; SV40, simian virus 40.

*To whom reprint requests should be addressed. E-mail: yosef.shaul@weizmann.ac.il.

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insoluble pellet was discarded, and the protein concentration was determined by using Bradford reagent (Bio-Rad). Equal amounts of protein were mixed with Laemmli sample buffer (4% SDS/20% glycerol/10% 2-mercaptoethanol/0.125 M Tris·HCl), heated at 95°C for 5 min, and loaded onto an 8% polyacrylamide-SDS gel. After electrophoresis, proteins were transferred to 0.45-μm cellulose nitrate membranes (Schleicher & Schuell). Loading equivalence and transfer efficiency were monitored by Ponceau S staining, and the membranes were then incubated with appropriate antibodies to proteins of interest followed by horseradish peroxidase-conjugated anti-IgG antibodies. Signals were developed by using Super Signal (Pierce) at 20°C for 5 min, and the membranes were then exposed to x-ray film (Fuji) for an appropriate time and developed. Membranes were stripped with 50 mM citric acid before a different primary antibody was used. The antibodies used were monoclonal anti-human p53 (Pab 1801) (26), monoclonal anti-mouse and human p53 (Pab240), monoclonal anti IκB (Santa Cruz Biotechnology), monoclonal anti-β-tubulin, and anti-HA (Sigma).

Apoptosis and Cell Viability Assays. Apoptosis in normal thymocytes was induced by γ-irradiation at 4 Gy (Co^{60} source, 0.63 Gy/min) and in M1-t-p53 cells by culture at 32°C. The percentage of apoptotic thymocytes was determined on May–Grünwald–Giemsa-stained cytospin preparations by counting 400 cells 5 h after γ-irradiation. Apoptotic cells were scored by their smaller size, condensed chromatin, and fragmented nuclei compared with nonapoptotic cells. Analysis of DNA fragmentation during apoptosis in thymocytes was performed by DNA agarose gel electrophoresis as described (27). Apoptotic M1-t-p53 cells undergo secondary changes, including uptake of Trypan Blue (28). The percentage of viable cells (nonapoptotic and not stained with Trypan Blue) was determined by counting 400 cells in a hemacytometer after 23 h at 32°C.

Results

Regulation of p53 Degradation by NQO1. To determine whether the p53 level is regulated by NQO1 in HCT116 human colon carcinoma cells expressing wild-type *p53* (29), cells were treated with the NQO1 inhibitor dicoumarol. Treatment for 90 min resulted in a significant reduction in p53 level, and after 180 min p53 was almost completely eliminated (Fig. 1*A*). To further test whether dicoumarol can similarly affect the p53 level accumulated upon irradiation, cells were γ-irradiated at 6 Gy and incubated for 4 h without or with dicoumarol. As expected, p53 accumulated upon γ-irradiation (compare Fig. 1*B*, lanes 1 and 2). Accumulation of p53 was lower in the presence of 200 μM dicoumarol and almost reached the uninduced level at 400 μM dicoumarol (Fig. 1*B*). Under the same conditions the level of β-tubulin was unaffected (Fig. 1*A* and *B*, *Bottom*), indicating that there is some specificity of this effect. To further establish the specific effect of dicoumarol on the p53 level, we used COS 1 cells in which p53 is stabilized because of expression of SV40 large T antigen (30). In contrast to the results obtained with HCT116 cells, the p53 level in COS 1 cells was not reduced by treatment with dicoumarol, even at 400 μM (Fig. 1*C*). The results indicate that dicoumarol caused a strong decrease in both basal and induced p53 levels. Because the NQO1 inhibitor could not overcome the p53 stabilization by SV40 large T antigen, we conclude that dicoumarol induces p53 protein destabilization.

It is well documented that p53 accumulation is determined by the rate of its proteasomal degradation (reviewed in refs. 1 and 2). To determine whether the observed p53 destabilization by dicoumarol occurs through protein degradation, cells were treated with dicoumarol together with the proteasome inhibitors MG132 or lactacystin. The results show that dicoumarol-induced p53 elimination was completely blocked by the addition of either

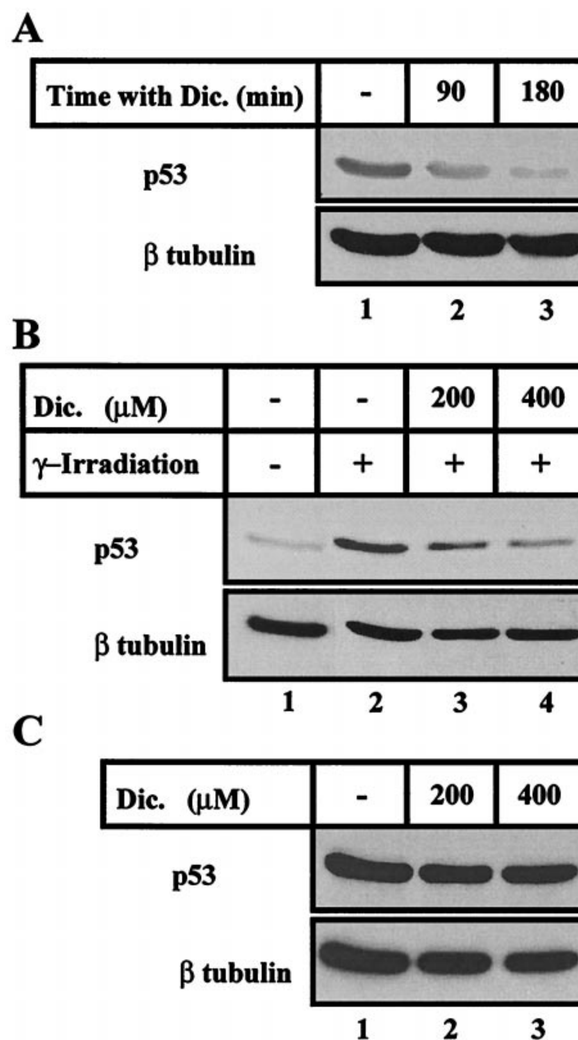

Fig. 1. Dicoumarol affects p53 stability. (*A*) HCT116 cells were incubated without (−) or with 400 μM dicoumarol for 90 and 180 min. (*B*) HCT116 cells were γ-irradiated at 6 Gy and incubated without (−) or with 200 and 400 μM dicoumarol for 4 h. This blot was underexposed compared with *A* to highlight the increase in p53 protein level after irradiation. (*C*) COS 1 cells were γ-irradiated at 6 Gy and incubated without (−) or with 200 and 400 μM dicoumarol for 4 h. Protein extraction and immunoblot analysis were carried out as described in *Materials and Methods*, with the use of Pab 1801 monoclonal anti-p53 antibody. The blots were then stripped and reprobed with monoclonal anti-β-tubulin antibody as a control for equal protein loading in each lane. Dic., dicoumarol.

MG132 (Fig. 2*A*) or lactacystin (Fig. 2*B*). These results indicate that dicoumarol induced p53 proteasomal degradation.

To verify that enhanced p53 degradation by dicoumarol was mediated by inhibition of NQO1 activity, we determined whether overexpression of *NQO1* could prevent dicoumarol-induced p53 degradation. A pool of stable clones of cells overexpressing HA-tagged *NQO1* was established, and the level of NQO1 protein was verified by immunoblotting. These cells became resistant to p53 degradation by dicoumarol (Fig. 3). Similar results were obtained with three individual stable clones expressing HA-tagged *NQO1*. The level of HA-NQO1 was not reduced in the presence of dicoumarol (Fig. 3). These results indicate that dicoumarol-induced p53 degradation is most probably the direct outcome of inhibition of NQO1 activity.

Suppression of p53-Mediated Apoptosis by Dicoumarol-Induced p53 Degradation. Induction of wild-type p53 accumulation in various cell types by overexpression of *p53* or by γ-irradiation can lead

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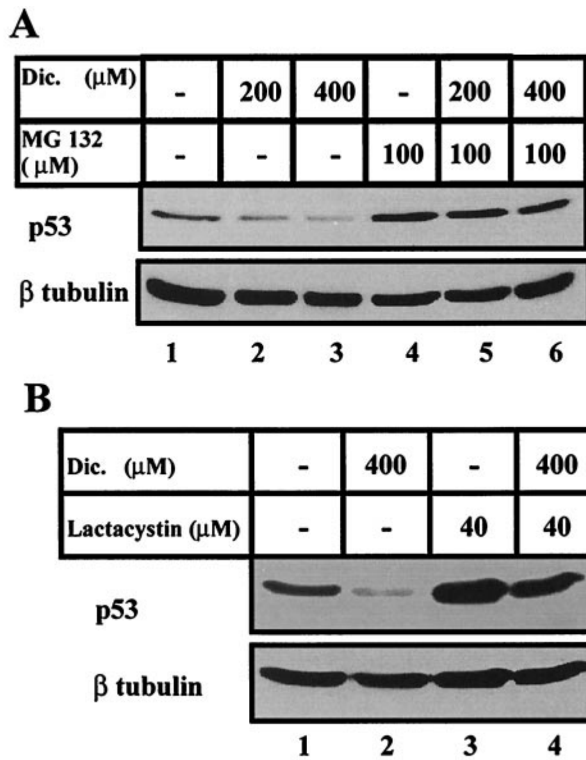

Fig. 2. Dicoumarol-induced p53 decrease occurs by proteasomal degradation. (*A*) HCT116 cells were incubated without (−) or with 200 and 400 μM dicoumarol and without (−) or with 100 μM MG 132 for 4 h. (*B*) HCT116 cells were incubated without (−) or with 400 μM dicoumarol without (−) or with 40 μM lactcystin for 4 h. Dic., dicoumarol.

to apoptotic cell death (3–6). To determine whether inhibition of p53 protein accumulation by the inhibition of NQO1 activity can also prevent apoptosis, we measured the effect of dicoumarol on p53-dependent apoptosis in γ-irradiated normal mouse thymocytes. Dicoumarol inhibited the induction of apoptosis in 4 Gy γ-irradiated thymocytes in a dose-dependent manner, as determined by morphological analysis of apoptosis (Fig. 4*A*) and DNA fragmentation at internucleosomal sites (Fig. 4*B*). Complete inhibition of apoptosis was obtained with 200 μM dicoumarol. The inhibition of p53-dependent apoptosis in γ-irradiated thymocytes by dicoumarol was associated with a decrease in the level of p53 (Fig. 4*C*). The results indicate that dicoumarol inhibits p53-mediated apoptosis in γ-irradiated normal thymocytes through enhanced p53 degradation.

We also determined the ability of dicoumarol to affect induction of apoptosis in M1-t-p53 myeloid leukemic cells that overexpress a temperature-sensitive *p53* transgene. These cells are viable and proliferate at 37°C when p53 behaves like a mutant form, but undergo apoptosis at 32°C when p53 behaves like wild type (3). Dicoumarol also inhibited p53-induced apoptosis in these cells. The addition of 75 μM or 100 μM dicoumarol at 32°C for 23 h resulted in an increase in cell survival compared with cells cultured under the same conditions without dicoumarol (Fig. 5*A*). Doses of 125 μM dicoumarol or more were toxic to these cells. As in HCT116 cells and normal thymocytes, the p53

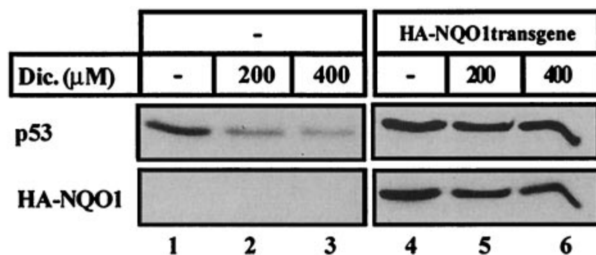

Fig. 3. Dicoumarol-induced p53 degradation is inhibited by overexpression of *NQO1*. Parental HCT116 cells (−) and a pool of HCT116 stable clones overexpressing HA-tagged *NQO1* were incubated without (−) or with 200 and 400 μM dicoumarol for 4 h. Immunoblot analysis was carried out with Pab 1801 monoclonal anti-p53 antibody, and the blots were then stripped and reprobed with monoclonal anti-HA antibody as a control for *NQO1* expression.

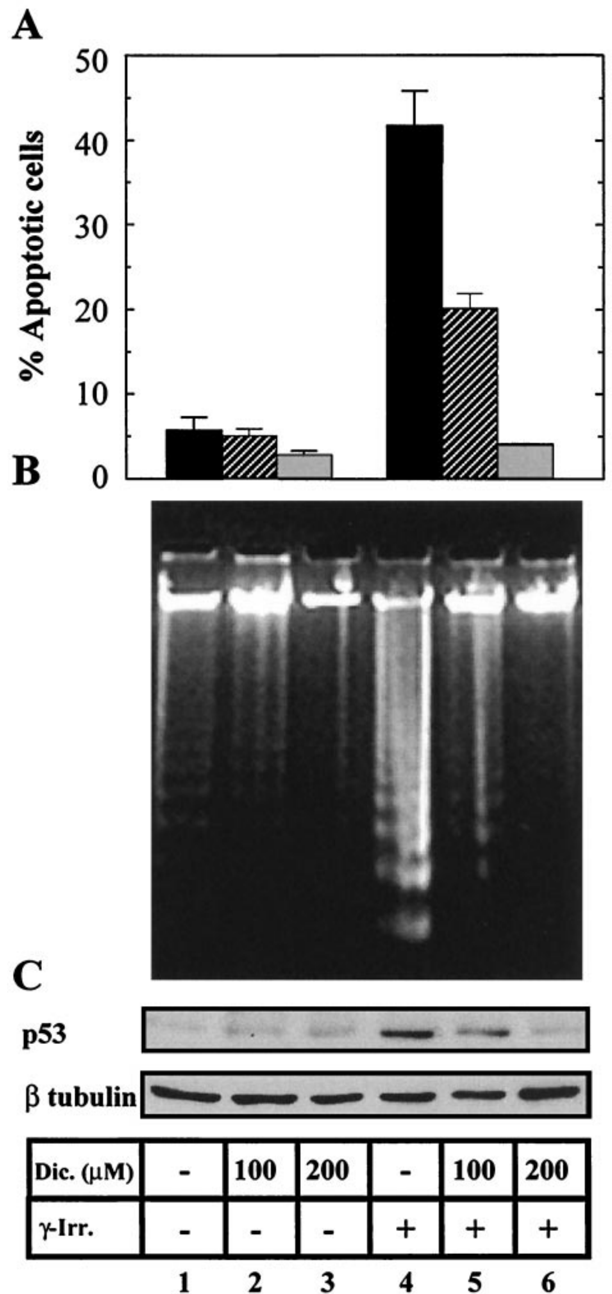

Fig. 4. Dicoumarol inhibits p53 accumulation and p53-dependent apoptosis in γ-irradiated thymocytes. Thymocytes that were not irradiated (−) or were γ-irradiated at 4 Gy (+) were cultured for 5 h without (−) or with 100 and 200 μM dicoumarol. (*A*) The percentage of apoptotic cells was determined on May–Grünwald–Giemsa-stained cytospin preparations. (*B*) DNA fragmentation at internucleosomal sites. (*C*) Immunoblot analysis was carried out with Pab 240 monoclonal anti-p53 antibody. Dic., dicoumarol; γ-irr., γ-irradiation.

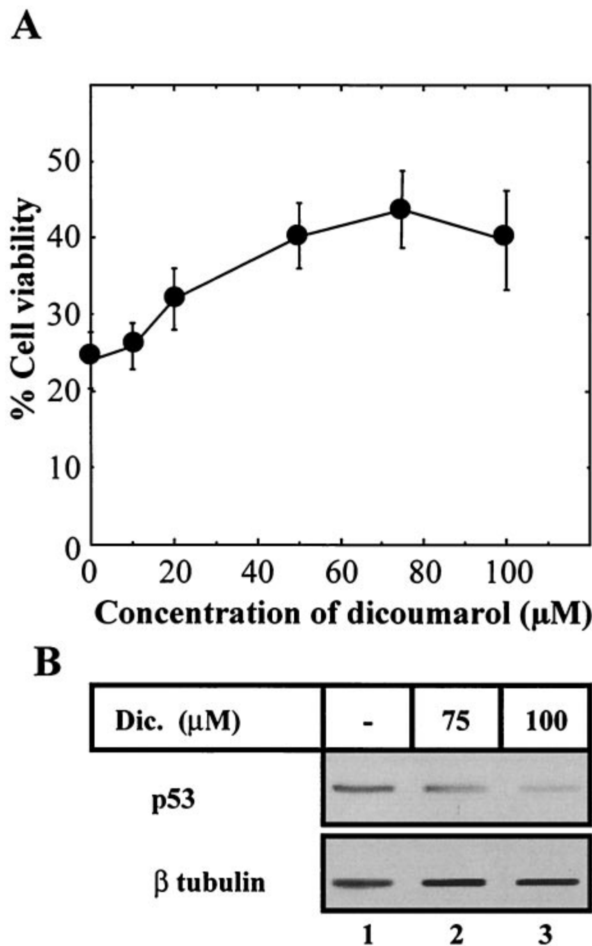

Fig. 5. Dicoumarol decreases the p53 level and p53-dependant apoptosis in M1-t-p53 cells. (*A*) M1-t-p53 myeloid leukemic cells were cultured at 32°C without or with different concentrations of dicoumarol, and the percentage of viable cells was determined after 23 h. Concentrations of dicoumarol above 125 μM were toxic to these cells. (*B*) Immunoblot analysis of the p53 level in cells cultured at 32°C for 16 h without (−) or with 75 or 100 μM dicoumarol was carried out with Pab 240 monoclonal anti-p53 antibody. Dic., dicoumarol.

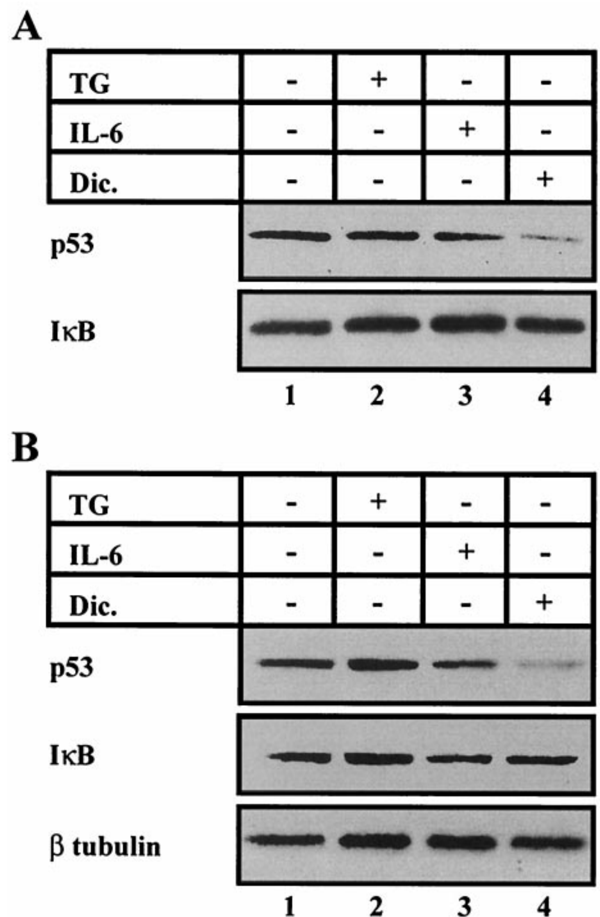

Fig. 6. Degradation of mutant and wild-type p53 by dicoumarol but not by other antiapoptotic agents such as IL-6 or thapsigargin. M1-t-p53 cells were cultured for 6 h without (−) or with 10 nM thapsigargin, 50 ng/ml IL-6, or 100 μM dicoumarol. (*A*) At 32°C, when p53 behaves like wild type. (*B*) At 37°C, when p53 behaves like a mutant. Immunoblot analysis was carried out with Pab 240 monoclonal anti-p53 antibody. The blots were then stripped and re-probed with anti-IκB and anti-β-tubulin antibody. Dic., dicoumarol.

level in M1-t-p53 cells cultured at 32°C was reduced by the addition of dicoumarol (Fig. 5*B*). Inhibition of NQO1 activity thus enhanced the degradation of overexpressed p53 and resulted in reduced p53-dependent apoptosis in these cells.

We previously have shown that IL-6 and the calcium-mobilizing compound thapsigargin are highly efficient antiapoptotic agents in M1-t-p53 cells (3, 31). The effect of these compounds on p53 stability was therefore also investigated. In contrast to dicoumarol, IL-6 and thapsigargin did not cause a reduction in p53 level in these cells at 32°C (Fig. 6*A*). Dicoumarol did not affect the level of IκB (Fig. 6*A*, *Bottom*). The ability of dicoumarol to induce degradation of p53 but not of IκB or β-tubulin also was observed in M1-t-p53 cells at 37°C, where the cells express a high level of mutant *p53* (Fig. 6*B*).

Discussion

We describe here a pathway regulating p53 accumulation. Accumulation of wild-type p53 after DNA damage (4–6) or overexpression even without prior DNA damage (3) induces apoptosis. Therefore, to avoid uncontrolled induction of apoptosis, the endogenous p53 protein is maintained at low levels by mechanisms that ensure its short half-life and degradation through the ubiquitin–proteasome pathway (32). A major component in this process is Mdm-2, which acts as a ubiquitin ligase that mediates p53 degradation in the proteasome (13, 14). Because *mdm-2* is a transcriptional target gene of p53 (33, 34), this system creates an autoregulatory negative loop that maintains cellular integrity. After DNA damage or other types of stress, p53 levels are increased through different posttranslational modifications of both p53 and Mdm-2 that reduce their association and thus decrease p53 degradation. These modifications include phosphorylations (35–38), acetylations (39), and interactions with proteins such as the DNA helicase WRN (40). In addition, the level and activity of Mdm-2 itself are modulated by other proteins, including the Mdm-2 antagonist p19^{ARF} (41, 42), or by Ras, which can enhance *mdm-2* transcription through the Raf–MEK–mitogen-activated protein kinase pathway (43). Our data provide experimental evidence for a role of NQO1 activity in p53 accumulation and thus establish a new pathway determining the p53 level. It will be interesting to determine whether this pathway is Mdm2-dependent.

We now show that endogenous and γ-irradiation-induced p53 levels were significantly reduced in different cell types by the NQO1 inhibitor dicoumarol. The ability of proteasome inhibitors to block this effect indicates that dicoumarol enhanced p53 proteasomal degradation. To confirm that p53 degradation enhanced by dicoumarol was due to inhibition of NQO1 activity,

we established cell lines that overexpress *NQO1*. These cells became resistant to dicoumarol-induced p53 degradation, indicating that the effect of dicoumarol was indeed due to inhibition of NQO1 activity. However, even inhibition of NQO1 by dicoumarol could not cause degradation of p53 that had already been stabilized by SV40 T antigen. This finding indicates that the NQO1 pathway for the regulation of the cellular p53 level is subject to regulation by other pathways that control p53 stability.

As shown with p53, NQO1 inhibition by dicoumarol also enhanced the degradation of p73 and c-Myc (data not shown). In contrast, NQO1 inhibition did not cause degradation of β-tubulin, NQO1, IκB, or poly(ADP-ribose) polymerase [data not shown for poly(ADP-ribose) polymerase]. These data attribute a certain level of specificity to this regulatory pathway of protein degradation. The proteins that were destabilized by dicoumarol, p53, p73, and c-Myc, share the capacity to induce apoptosis (3–6, 44–47). However, NQO1 inhibition also destabilized a mutant p53, which does not induce apoptosis. It will be interesting to determine whether the stability of other apoptosis-regulating proteins is also subject to regulation by NQO1 activity and whether dicoumarol can inhibit p73-mediated and c-Myc-mediated apoptosis pathways.

NQO1 is a ubiquitous flavoenzyme that catalyzes two-electron reduction of various quinones, with NADH or NADPH as an electron donor. The involvement of NQO1 in p53 accumulation suggests that redox reactions controlled by oxidoreductases may be an important factor in determining the p53 intracellular level. The recent finding that overexpression of another oxidoreductase, WOX1, can increase the p53 protein level in L929 cells (48) supports this suggestion. p53 accumulates in cells under hypoxic conditions when the cellular level of reactive oxygen species is low (reviewed in ref. 49). p53 also accumulates after DNA damage that is associated with increased production of reactive oxygen species (reviewed in refs. 2 and 49). Because both oxidants as well as antioxidants up-regulate the expression of NQO1 (18), it can be suggested that one mechanism that regulates p53 accumulation under irradiation or hypoxic conditions is mediated by increased NQO1 activity. Some p53-inducible genes encode proteins that may participate or respond to oxidative stress, including p53-inducible gene 3 (15) and glutathione peroxidase (50). Our sequence analysis of the NQO1 promoter has revealed a putative p53-binding element. We therefore suggest that NQO1, glutathione peroxidase, and p53-inducible gene 3 may be part of a positive autoregulatory loop that regulates the level of p53.

The oxidoreductase activity of NQO1 is mediated by the conversion of NADH to NAD^+. Therefore, inhibition of NQO1 by dicoumarol might cause a substantial NAD^+ loss. NAD^+ serves as a substrate for poly(ADP-ribose) polymerase, a DNA-binding protein that catalyzes the transfer of ADP-ribose residues from NAD^+ to itself and to other proteins, including p53 (reviewed in ref. 51). Human breast, skin, and lung cells with reduced NAD level due to the use of nicotinamide-deficient medium, as well as cells deficient in poly(ADP-ribose) polymerase, exhibit decreased basal levels of p53 protein (52, 53). In addition, cells with defective NAD/poly(ADP-ribose) polymerase metabolism showed resistance to DNA damage-induced p53 accumulation and subsequent apoptosis (53). Therefore, dicoumarol-induced loss of NAD^+ can provide a mechanism for p53 regulation by NQO1. Recently, NAD has been shown to play an important role in the regulation of gene expression and life span in yeast (54–56). A major gene in this process is Sir2p, which possesses ADP-ribosyltransferase activity and promotes silencing of gene transcription at selected loci (54, 55). In yeast increased longevity can be induced by calorie restriction, and this effect requires Sir2p and one of the two major pathways of NAD synthesis (56). Sir2p and p53 are structurally and functionally distinct, and our results suggest that they share the capacity to regulate cell fate via NAD (Fig. 7).

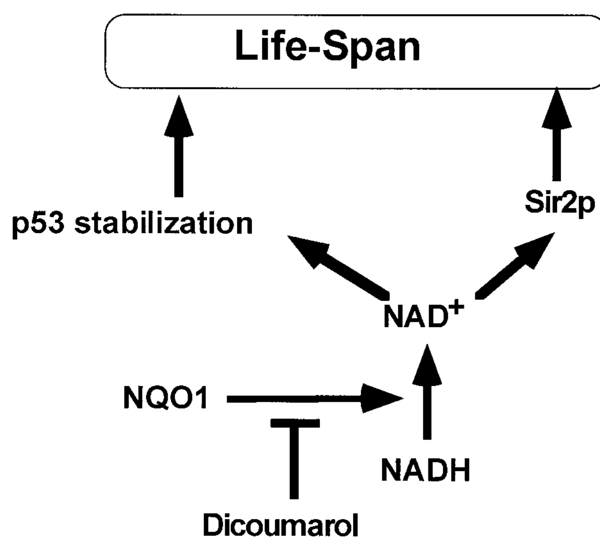

Fig. 7. Model of the role of NQO1 in p53 stabilization and life span. It is assumed that NQO1 determines the level of NAD^+ and that this regulates the level of p53. The stabilization of p53 results in either apoptosis or growth arrest, which regulate life span. Also shown is the NAD^+–Sir2p pathway that regulates life span in yeast (56).

Mutations in *p53* are found in more than 50% of cases of human cancer (1, 2). Some *p53* mutants are gain-of-function mutants, which can suppress apoptosis (57–59). The ability of dicoumarol to induce the degradation of p53 in its mutant form and the fact that dicoumarol is already in clinical use as an anticoagulant raises the possibility of combining dicoumarol with cytotoxic agents in therapy against cancer cells that express high levels of mutant *p53*.

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