MDM2 and MDMX bind and stabilize the p53-related protein p73

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The p53 gene encodes one of the most important tumor suppressors in human cells and undergoes frequent mutational inactivation in cancers. MDM2, a transcriptional target of p53, binds p53 and can both inhibit p53-mediated transcription [1,2] and target p53 for proteasome-mediated proteolysis [3,4]. A close relative of p53, p73, has recently been identified [5,6]. Here, we report that, like p53, p73α and the alternative transcription product p73β also bind MDM2. Interaction between MDM2 and p53 represents a key step in the regulation of p53, as MDM2 promotes the degradation of p53. In striking contrast to p53, the half-life of p73 was found to be increased by binding to MDM2. Like MDM2, the MDM2-related protein MDMX also bound p73 and stabilized the level of p73. Moreover, the growth suppression functions of p73 and the induction of endogenous p21, a major mediator of the p53-dependent growth arrest pathway, were enhanced in the presence of MDM2. These differences between the regulation of p53 and p73 by MDM2/MDMX may highlight a physiological difference in their action.

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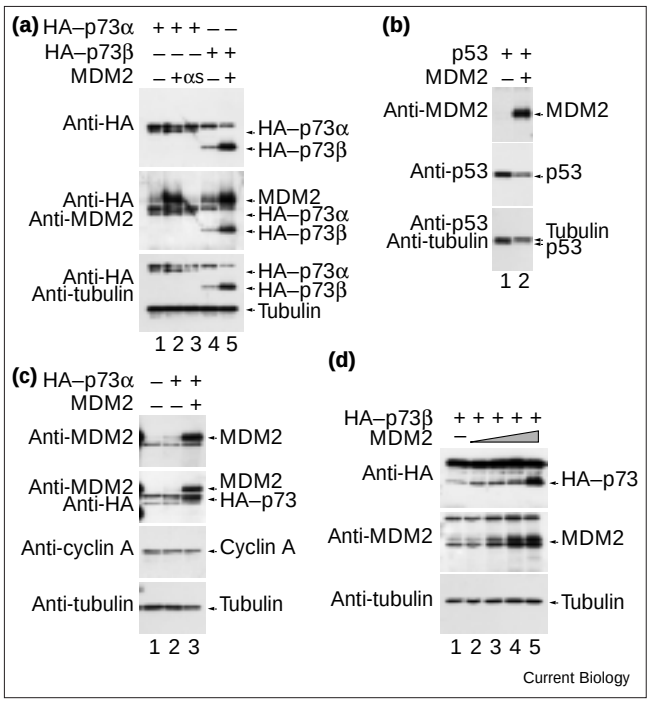
Figure 1



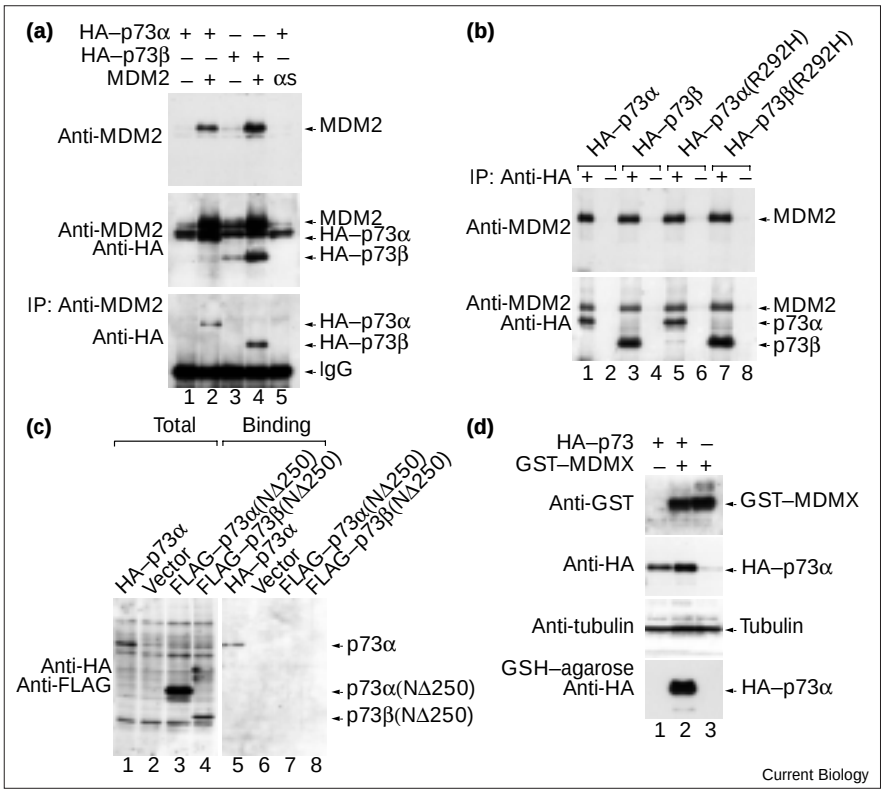
MDM2 destabilizes p53 but stabilizes p73. **(a)** Embryonic kidney 293 cells were transfected with plasmids expressing HA–p73α, HA–p73β, MDM2 and antisense MDM2 (αs) in the indicated combinations. Cells were grown for a further 24 h after transfection and then harvested. Cell extracts were prepared, electrophoresed on 7.5% SDS–polyacrylamide gels and immunoblotted with antibodies against HA, HA and MDM2, or HA and tubulin. The identity of the p73α bands was confirmed using an antibody raised against a carboxy-terminal peptide of p73α (data not shown). The positions of HA–p73α, HA–p73β, MDM2 and tubulin are indicated. **(b)** The plasmid expressing p53 was co-transfected with a control plasmid (lane 1), or with a plasmid expressing MDM2 (lane 2) into 293 cells. Cell extracts were subjected to immunoblotting for MDM2, p53, or p53 and tubulin. **(c)** Extracts from SAOS-2 cells transfected with plasmids expressing HA–p73α (lanes 2,3) and MDM2 (lane 3) were subjected to immunoblotting with antibodies against MDM2, or MDM2 and HA. Equal loading of samples was verified by immunoblotting with antibodies against cyclin A or tubulin. **(d)** H1299 cells were transfected with a plasmid expressing HA–p73β (4 μg), and an increasing amount of plasmid expressing MDM2 (lanes 1–5: 0 μg, 2 μg, 4 μg, 10 μg, 12 μg, respectively). Cell extracts were subjected to immunoblotting for HA, MDM2 or tubulin.

Results and discussion

MDM2 destabilizes p53 but stabilizes p73

Plasmids expressing MDM2 and hemagglutinin (HA)-tagged versions of p73α and p73β (HA–p73α and HA–p73β) were co-transfected into 293 cells. The expression levels of MDM2 and HA–p73 were detected by immunoblotting with specific antibodies (Figure 1a). Surprisingly, the levels of both p73α and p73β were much higher when co-transfected with MDM2, compared with cells transfected with p73 alone. Figure 1a also shows the decrease in MDM2, and the lack of p73α elevation, in cells transfected with the antisense MDM2 plasmid (lane 3). Immunoblotting the same membrane for tubulin showed similar loading of samples. As expected, co-expression of

Figure 2



Interaction between p73 and MDM2 or MDMX. (a) HA–p73α, HA–p73β, MDM2 and antisense MDM2 (αs) were expressed in 293 cells in the indicated combinations. Cell extracts were subjected to immunoblotting with antibodies against MDM2, or MDM2 and HA. Cell extracts (100 μg) were immunoprecipitated (IP) with an anti-MDM2 antibody, and immunoblotted with an anti-HA antibody (bottom panel). The positions of p73α, p73β, MDM2 and immunoglobulin G (IgG) heavy chains from the immunoprecipitation are indicated. (b) MDM2 was co-expressed in 293 cells with HA–p73α, HA–p73β, HA–p73α(R292H) and HA–p73β(R292H). Cell extracts (100 μg) were immunoprecipitated with either anti-HA antibody or an unrelated antibody as indicated. The immunoprecipitates were immunoblotted for MDM2, or MDM2 and HA. (c) A plasmid encoding GST–MDM2 was co-transfected into HtTA1 cells with plasmids expressing HA–p73α, FLAG-epitope-tagged p73α(NΔ250) or p73β(NΔ250), or vector controls. Cell extracts (100 μg) were incubated with GSH–agarose to isolate GST–MDM2 and associated proteins as described [10]. Total cell lysates (lanes 1–4) and the GSH–agarose precipitates (lanes 5–8) were immunoblotted for HA and FLAG. (d) HA–p73α and GST–MDMX were expressed in 293 cells in the indicated combinations. Cell extracts were prepared and subjected to immunoblotting for GST, HA or tubulin. In the bottom panel, cell extracts (100 μg) were incubated with GSH–agarose as above, and immunoblotted for HA.

MDM2 with p53 reduced the level of p53 (Figure 1b). Immunoblotting for p53 and tubulin together clearly indicated that, although the level of tubulin remained constant, p53 levels decreased when MDM2 was present.

High transfection efficiency was achieved in 293 cells, but these cells contained endogenous p53. To rule out the involvement of p53 in these experiments, we also used the p53-null SAOS-2 and H1299 cells. Co-expression of MDM2 increased the level of p73 in SAOS-2 cells (Figure 1c, only p73α is shown). Moreover, a progressive increase in p73 was seen when increasing amounts of MDM2 were co-transfected into H1299 cells (Figure 1d; only p73β is shown). Interestingly, transfection of p73α increased the level of endogenous MDM2, suggesting that p73α can also activate MDM2 transcription. These results clearly demonstrate that p73 can be stabilized by MDM2 under conditions in which p53 is destabilized, and suggest that the two closely related proteins can be differentially regulated.

Association of p73 and transcriptionally inactive mutants of p73 with MDM2

As the sequence of the MDM2-binding domain of p53 (TFSDLW in the single-letter amino-acid code) is similar to that of p73 (TFEHLW), we next investigated whether MDM2 could form a complex with p73. Figure 2a shows that, when co-expressed with MDM2, both p73α and p73β could be detected in MDM2 immunoprecipitates. This result was verified in the reciprocal experiment in which MDM2 was found in HA–p73 immunoprecipitates but not in control immunoprecipitates (Figure 2b). Figure 2b also shows that the transcriptionally inactive mutants of p73α and p73β, in which the Arg292 residue was mutated to His (R292H), could also bind MDM2, suggesting that the transcriptional activity of p73 was not required for the binding. To determine whether binding of p73 to MDM2 was involved in the stabilization of p73 (see below), we created amino-terminal truncation mutants (NΔ250) of p73α and p73β and tested their binding to MDM2. Figure 2c shows that only full-length p73α, but not NΔ250, could bind to MDM2 tagged with glutathione S-transferase (GST). In support of some of the findings described here, interaction between MDM2 and p73 has also been reported recently [7,8].

Binding and stabilization of p73 by MDMX

MDMX is structurally similar to MDM2 but is not subject to transcriptional activation by p53 [9]. A GST–MDMX

fusion was co-expressed with p73 in mammalian cells. Figure 2d (only p73α is shown here) shows that p73α was stabilized when co-expressed with GST–MDMX. To study the possible interaction between p73 and MDMX, cell extracts were incubated with agarose coupled to reduced glutathione (GSH–agarose) to precipitate GST–MDMX and any associated p73α. From Figure 2d, it is apparent that p73α was only precipitated when co-expressed with GST–MDMX. These data suggest that MDMX, like MDM2, can bind to and stabilize p73.

MDM2 affects the half-life of p73

MDM2 regulates the level of p53 by targeting p53 for proteasome-dependent degradation [3,4]. To determine whether the elevation in p73 levels by MDM2 was due to an increase in transcription of p73, we assessed the levels of HA–p73α mRNA by RT–PCR. Figure 3a shows that the relative level of HA–p73α mRNA was similar in the presence or absence of MDM2, suggesting that the effect of MDM2 on p73 may be post-translational. Figure 3b shows that, after treatment with LLnL, a proteasome/calpain inhibitor, more HA–p73β was detected, suggesting p73β may be degraded by a proteasome-dependent pathway. Significantly, LLnL did not further increase the level of p73β in the presence of MDM2, suggesting that MDM2 and LLnL may both act on the same targets to promote p73 stability (that is, prevent proteasome-mediated degradation).

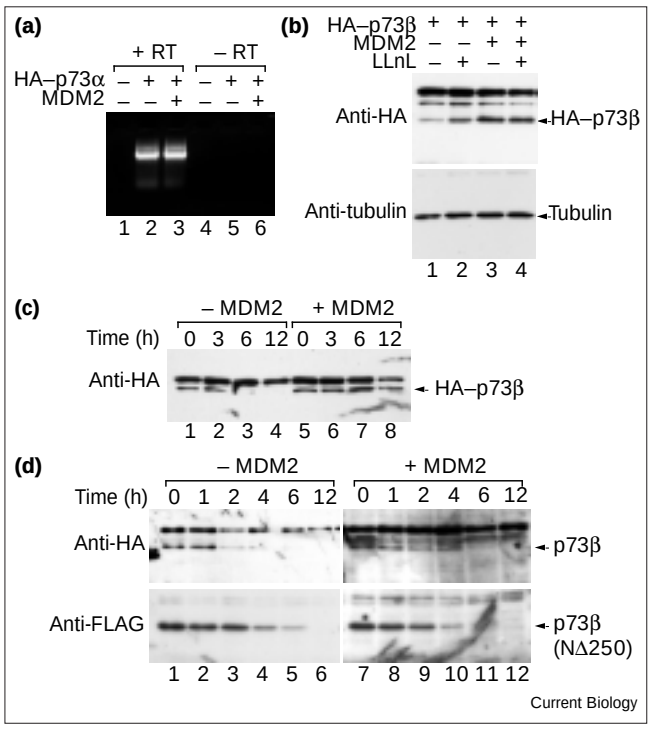
For an initial idea of the half-life of p73, expression of p73 was turned off using cycloheximide (Figure 3c); we found that p73 was more stable when co-expressed with MDM2. For a better indication of the half-life, expression of p73 and NΔ250 truncation mutants were put under the control of deoxycycline. Figure 3d shows that, when co-expressed with MDM2 (constant expression not under deoxycycline control), the half-life of p73β was appreciably longer than the control. In contrast, the half-life of the p73β (NΔ250) mutant was not affected by MDM2, suggesting that the stabilization of p73 required interaction with MDM2. Similarly, we found that amino-terminal deletion mutants of MDM2 did not bind p53/p73, nor affect the protein level of p53/p73 (our unpublished observations). Taken together, these data suggest that the rate of degradation of p73 can be decreased by binding to MDM2.

MDM2 enhances the anti-proliferative function of p73

We next investigated whether the biological functions of p73 were influenced by MDM2. Figure 4a shows that the endogenous p21 was induced by p73α but not by the transcriptionally inactive R292H mutant. Significantly, co-expression of MDM2 with p73α enhanced the induction of p21. The growth potential of cells expressing p73 and MDM2 was investigated by colony formation assays. Expression of p73α reduced the number of colonies to about 40% of the control (Figure 4b). Co-expression of p73α with MDM2 further reduced the number of the colonies (to about 15%). Our unpublished data also indicate that co-expression of p73 and MDM2 increased the sub-G1 DNA population in HeLa cells, suggesting that induction of apoptosis by p73 was enhanced by MDM2.

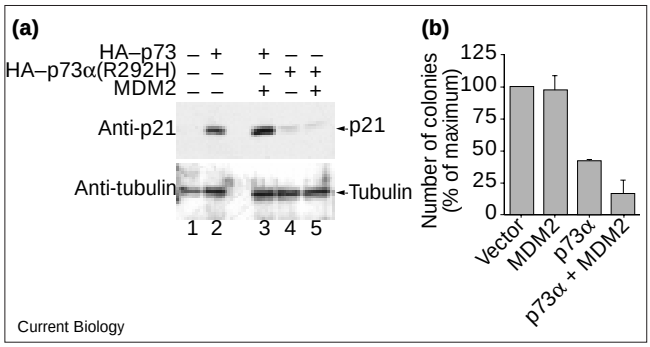
There are two distinct ways in which MDM2 can inactivate p53. MDM2 can inhibit p53-mediated transcription by masking the transactivating domain [1,2], as well as targeting p53 for proteolysis [3,4]. Preliminary evidence

Figure 3



Regulation of p73 protein level by MDM2. (a) Embryonic kidney 293 cells were transfected with the indicated combinations of plasmids expressing HA–p73α and MDM2. The relative amount of HA–p73α mRNA was analyzed by reverse transcriptase (RT)–PCR using SP6 and T7 oligonucleotides. RT was excluded in lanes 4–6. (b) A plasmid expressing HA–p73β was transfected into H1299 cells with a vector control or with a plasmid expressing MDM2. After transfection, the cells were divided into two identical plates (lanes 1,2: vector; lanes 3,4: MDM2) and allowed to grow for 24 h. Buffer (lanes 1,3) or LLnL (100 μM; lanes 2,4) was added to the medium, and the cells incubated for another 12 h. Cell extracts were subjected to immunoblotting for HA or tubulin. (c) A plasmid expressing HA–p73β was co-transfected with a vector control (lanes 1–4) or with a plasmid expressing MDM2 (lanes 5–8) into H1299 cells. Cycloheximide (10 μg/ml) was added to the medium, and the cells were harvested at the indicated times. Cell extracts were subjected to immunoblotting for HA. (d) Plasmids carrying HA–p73β and FLAG–p73β(NΔ250) under inducible control of deoxycycline were co-transfected into HtTA1 cells with a control plasmid (lanes 1–6) or with a plasmid expressing MDM2 (lanes 7–12). At 24 h after transfection, deoxycycline (1 μg/ml) was added to the medium to turn off the expression of p73β and p73β(NΔ250). The cells were harvested at the indicated times for extract preparation, and immunoblotted for HA or FLAG.

Figure 4



Inhibition of cell proliferation by p73 and MDM2. (a) H1299 cells were transfected with plasmids expressing the CD2 surface marker (5 μg) together with that expressing HA–p73α or HA–p73α(R292H) (5 μg), or MDM2 (10 μg) in the combinations indicated. Transfected cells were isolated by selection with magnetic beads coupled to anti-CD2 antibody. Cell extracts were prepared and immunoblotted with anti-p21 or anti-tubulin antibody. (b) H1299 cells were transfected with a plasmid that conferred puromycin-resistance, together with the control vector, plasmids expressing MDM2 (10 μg), HA–p73α (6 μg), or HA–p73α and MDM2 together, as indicated. The colony formation assay was as described in Materials and methods. The average of three independent experiments and the standard deviation are shown. Note that the inhibition of colony formation by p73 was not 100% because the puromycin-resistance gene and the p73 DNA were on different plasmids, and only the integration of the puromycin-resistance plasmid was selected for.

indicated that MDM2 could also mask the transactivating domain of p73, depending on the promoter and the cell line used (our unpublished data). The relative importance of the stabilization of p73 versus the transcriptional masking by MDM2 is currently under investigation.

Materials and methods

DNA constructs

HA–p73 constructs in pcDNA3 were from W.G. Kaelin Jr. (Dana-Farber Cancer Institute) [5]. FLAG-tagged NΔ250 in pUHD-P1 was constructed by putting the *Eco*RI–*Xba*I fragments of p73 in pcDNA3 into pUHD-P1 [10]. HA–p73 in pUHD-P1 was constructed by putting the *Sac*II–*Eco*RI fragment of p73α or p73β in pcDNA3 into FLAG–p73(NΔ250) in pUHD-P1. MDM2 in pCMV was a gift from B. Vogelstein (Johns Hopkins Oncology Center), and murine p53 in pCMV from T. Hunter (Salk Institute). Human MDMX was amplified by RT–PCR from A549 cells, and subcloned into pCAGGS vector for expression of GST–MDMX. The β-galactosidase construct was from Y. Chen (Salk Institute), CD2 construct was from C. Norbury (University of Oxford), and the CD20 construct was as described [10].

Cell culture

Transformed embryonic kidney 293 cells, non-small-cell lung carcinoma H1299 cells and osteogenic sarcoma SAOS-2 cells were obtained from the American Type Culture Collection. HtTA1 cells (gift from H. Bujard) were HeLa cells (cervical carcinoma) stably transfected with pUHD15-1 [11]. Cells were transfected using the calcium phosphate precipitation method [12]. Unless stated otherwise, cells were transfected with 4 μg p73 DNA and 8 μg MDM2 DNA for 60 mm plates, and the total amount of DNA for each transfection was adjusted to the same level using vector DNA. Cells were grown for a further 24 h for 293 cells, 36 h for H1299 cells, or 48 h for SAOS-2 cells after transfection. Typical transfection efficiencies obtained were ~50% (293 and HeLa), 20% (H1299) and 5–10% (SAOS-2). Cell-free extracts were prepared as described [10]. For colony formation assays, 5×10^4 cells per 10 cm plate were transfected with a puromycin-resistant plasmid, and allowed to grow for 48 h before 2 μg/ml puromycin was added. After another 2 weeks, colonies were fixed with methanol:acetic acid (2:1 v/v) and visualized by staining with 2% w/v crystal violet. Around 500 colonies were counted for each experiment.

Antibodies and immunological methods

Mouse monoclonal antibody 421 against p53, E72 against cyclin A, rat monoclonal antibody YL1/2 against tubulin, and rabbit anti-GST antibody were from T. Hunt (ICRF, South Mimms). Monoclonal antibody 12CA5 against the HA was from T. Hunter; 2A10 against MDM2 was from A. Levine (Princeton University); M2 against FLAG was obtained from Eastman Kodak; OX-34 against CD2 was from C. Norbury (University of Oxford). Goat anti-p73α polyclonal antibodies (sc-7238) and rabbit anti-p21 antibodies (sc-397) were from Santa Cruz Biotechnology. Immunoblotting and immunoprecipitations were performed as described [10]. CD2-positive cells were selected by magnetic beads according to the manufacturer's instruction (DYNAL).

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