A Possible Role of p73 on the Modulation of p53 Level through MDM2

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ABSTRACT

MDM2, one of the transcriptional targets of p53, can target p53 for degradation in a negative feedback loop. The p53-related protein p73, however, can bind to MDM2 but is not consequently down-regulated. Here we demonstrate that p73 could transactivate the MDM2 promoter in p53-null cell lines. In p53-null cell lines, the level of MDM2 was increased by p73 due to increases in transcription and protein stability of MDM2. In transient transfection assays, inhibition of the transcriptional activity of p73 required a higher amount of MDM2 than that of p53. This is probably due to the fact that MDM2 can target p53, but not p73, for degradation. We demonstrated further that the level of p53 could be altered by a cooperation between MDM2 and p73, but not by transcriptional inactive mutants of p73. Expression of p73 resulted in a reduction of the ectopically expressed p53 in transient transfections or of the endogenous p53 induced by Adriamycin- or UV-mediated damage. These reductions of p53 were likely to be due to an increase in MDM2-mediated proteolysis. These results suggest the possibility that different levels of p73 in the cell may act as a mechanism to modulate p53 responses after DNA damage and other stresses and that an increase rather than a decrease in p73 may play a role in tumorigenesis.

INTRODUCTION

Loss of p53 tumor suppressor function is one of the most common steps in tumorigenesis of many types of cancer (1). The functions of p53 are primarily mediated through the regulation of cell cycle checkpoints and apoptosis. p53 is highly regulated at the levels of transcriptional activity, localization, and protein stability. Such tight control is provided by one of transcriptional targets of p53, MDM2, in a negative feedback loop. MDM2 can bind to the NH2-terminal transactivation domain of p53 and inhibit p53-mediated transcription (2–4). MDM2 can also shuttle p53 out of the nucleus by the virtue of the nuclear exporting signal in MDM2 (5–8). Furthermore, MDM2 reduces the level of p53 by targeting p53 for ubiquitin-mediated proteolysis (9, 10). In this connection, MDM2 itself functions as a ubiquitin ligase for p53 (11, 12).

The control of MDM2 over p53 is disrupted after DNA damage. After DNA damage, protein kinases like ATM or DNA-PK are activated and phosphorylate p53 at Ser-15 and other sites. These phosphorylations of p53 inhibit the binding of MDM2 to p53, therefore leading to an increase in p53 level, nuclear localization, and transactivation activity (13–15). This hypothesis elegantly explains how p53 level and activity are regulated by DNA damage.

MDM2 is overexpressed in a variety of tumors, which would serve to eliminate p53 responses after DNA damage or other stresses (16). The inhibition of p53 by MDM2 is subjected to regulation by ARF. ARF and the cyclin-dependent kinase inhibitor INK4A are gene products of the INK4A locus on human chromosome 9p21. ARF inhibits the degradation of p53 (17, 18) by binding to MDM2 and sequestering it into the nucleolus (19–21).

Several proteins that share a high degree of sequence similarity to p53 have been identified (22). These include p73 and its alternatively spliced forms (23–25) and p63 and its various variants (two of them were also cloned as p51A and p51B; Refs. 26–28). We and others have shown that, like p53, p73 can also bind to MDM2; however, unlike p53, binding of MDM2 to p73 does not target p73 for degradation (29–33). Similar to p53, p73 is also induced by certain types of DNA damage (34–36). Induction of p73 after DNA damage is dependent on phosphorylation of p73 on Tyr-99 by c-Ab1l (34–36). Interaction between p73 and c-Ab1l occurs through the SH3 domain of c-Ab1l and the COOH-terminal homo-oligomerization domain of p73. Recently, it has been shown that E2F-1 activates the transcription of p73 and induces apoptosis (37, 38).

Possible physiological functions of the p53-related proteins have been suggested by gene disruption studies in mice. Mice with disrupted p73 have neurological, phenomonal, and inflammatory defects (25). Unlike p53−/− mice, however, p73−/− mice do not develop tumors spontaneously. One interesting finding about the in vivo function of p73 is that a major form of p73 in the cell is a NH2-terminal-truncated form, which would be incapable of transcriptional activation (25). The p63 gene product appears to have important functions in development because disruption of p63 in mice affects limb and epitelial development (39, 40), and heterozygous germ-line mutations of p63 genes are found in human EEC syndrome (41).

Here we demonstrate that like p53, p73 could transactivate the MDM2 promoter in p53-null cell lines. The level of MDM2 was increased by p73 because of increases in the transcription and protein stability of MDM2. We demonstrated that the level of p53 could be altered by a cooperation between MDM2 and p73, but not with transcriptional inactive mutants of p73. Expression of p73 resulted in a decrease in exogenous p53 in transient transfection experiments or in endogenous p53 induced by DNA damage. These results suggest the possibility that p53 responses after DNA damage and other stresses can be modulated by different levels of p73.

MATERIALS AND METHODS

DNA Constructs. Constructs of HA4-tagged simian p73α and p73β and their transcriptional inactive mutants (R292H) in pcDNA3 were gifts from Dr. Daniel Caput (Sanofi Recherche, LaBege, France). MDM2 in pCMV was a gift from Dr. Arnold Levine (Princeton University, Princeton, NJ). The CD2 construct was a gift from Dr. Chris Norbury (ICRF, University of Oxford, Oxford, United Kingdom). MDM2 promoter-luciferase reporter constructs was a gift from Dr. Moshe Oren (The Weizmann Institute, Rehovot, Israel).

Cell Culture and Transfection. H1299 cells (human non-small cell lung carcinoma), MCF-7 (human mammary adenocarcinoma), SAOS-2 (human osteogenic sarcoma), and U2OS (human osteosarcoma) were obtained from the American Type Culture Collection (Manassas, VA). Cells were grown in 1598

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4 The abbreviations used are: HA, hemagglutinin; ICRF, Imperial Cancer Research Fund; CMV, cytomegalovirus.
DMEM supplemented with 10% v/v fetal bovine serum in a humidified incubator at 37°C with 5% CO₂. Semiconfluent cells were transiently transfected in a humidified incubator at 37°C with 5% CO₂. Semiconfluent cells were transiently transfected with the calcium phosphate precipitation method (42). Unless stated otherwise, 10 μg of each plasmid were used to transfect cells in 10-cm plates. The total amount of DNA for each transfection was adjusted to the same level regardless of the amount of MDM2 with p73 was not able to inhibit the transcriptional activity of p73 when the amount of MDM2-expressing plasmid was progressively raised (Fig. 1C), suggesting that the transcriptional activity of p73 could be inactivated by a higher concentration of MDM2. These data indicate that the MDM2 promoter can be transactivated by p73 in a p33-null background. Furthermore, inhibition of p73 appears to require a higher concentration of MDM2 than inhibition of p53, although both p73 and p53 were driven by similar CMV promoters. One explanation is from our previous data, which show that MDM2 binds to p73 but does not target p73 for degradation (31). Hence, at a low concentration of MDM2, p53 but not p73 is destabilized. However, at a high concentration of MDM2, both p53 and p73 are saturated with MDM2, and their transcriptional activities are inhibited.

We next studied whether the protein level of MDM2 is affected by expression of p73. Accumulation of endogenous MDM2 (both the M₇5, 55,000 and the M₉90,000 forms) was observed when p73[R292H] was overexpressed in SAOS-2 or H1299 cells (Fig. 2, Lanes 2 and 5). It is interesting that after transfection of p73, both the M₇5, 55,000 and M₉90,000 forms of endogenous MDM2 were seen in SAOS-2 cells (Lane 2), but only the M₉90,000 form was present in H1299 cells

**RESULTS**

Up-Regulation of MDM2 by p73 at the Levels of Transcription and Protein Stability. It was shown that the MDM2 promoter could be activated to varying degrees by p73 (29, 33, 47–49). But other studies have shown that p73 has no effect on the transcription of MDM2 (50). We therefore set out to initially determine whether p73 could activate the MDM2 promoter and whether this transcriptional activation is affected by MDM2. The p53-null lung carcinoma cell line H1299 was transfected with a reporter plasmid containing the MDM2 promoter placed upstream of luciferase. As expected, cotransfection of a p53 expression plasmid stimulated transcription from the MDM2 promoter (Fig. 1A). Cotransfection of the same amount of MDM2 plasmid inhibited the transcriptional activity of p53 by about 80%.

Fig. 1B shows that like p53, expression of p73α in H1299 cells transactivated the MDM2 promoter. In contrast to p53, cotransfection of the same amount of MDM2 with p73α did not result in an inhibition of the transcriptional activity of p73α. As a control, a transcriptional inactive mutant of p73α (R292H) was not able to transactivate the MDM2 promoter and was also not affected by MDM2. Similar results were seen with the alternatively spliced p73β, as well as in another p53-null cell line, SAOS-2 (data not shown). We found that transactivation of the MDM2 promoter by p73 was inhibited when the amount of MDM2-expressing plasmid was progressively raised (Fig. 1C), suggesting that the transcriptional activity of p73 could be inactivated by a higher concentration of MDM2. These data indicate that the MDM2 promoter can be transactivated by p73 in a p33-null background. Furthermore, inhibition of p73 appears to require a higher concentration of MDM2 than inhibition of p53, although both p73 and p53 were driven by similar CMV promoters. One explanation is from our previous data, which show that MDM2 binds to p73 but does not target p73 for degradation (31). Hence, at a low concentration of MDM2, p53 but not p73 is destabilized. However, at a high concentration of MDM2, both p53 and p73 are saturated with MDM2, and their transcriptional activities are inhibited.

We next studied whether the protein level of MDM2 is affected by expression of p73. Accumulation of endogenous MDM2 (both the M₇5, 55,000 and the M₉90,000 forms) was observed when p73α or p73β was overexpressed in SAOS-2 or H1299 cells (Fig. 2, Lanes 2 and 5). It is interesting that after transfection of p73, the M₇5, 55,000 and M₉90,000 forms of endogenous MDM2 were seen in SAOS-2 cells (Lane 2), but only the M₉90,000 form was present in H1299 cells.

**Antibodies and Immunological Methods.** Monoclonal antibody DO-1 against p53 (sc-126) and rabbit anti-p21(\textsuperscript{CIP1/WAF1}) antibodies (sc-397) were from Santa Cruz Biotechnology. Monoclonal antibody 2A10 against MDM2 was a gift from Dr. Arnold Levine. Ab-1 against MDM2 was from Oncogene Science, and 12CA5 against the HA tag was from Roche Molecular Biochemicals. Anti-CD2 (OX-34) conjugated to magnetic beads (DYNAL) was a gift from Dr. Chris Norbury; IF-6 antibody against topoisomerase I was a gift from Dr. Ian Hickson (ICRF, University of Oxford); and YL1/2 against tubulin was a gift from Dr. Tim Hunt (ICRF, South Mimms, United Kingdom). Immunoblottings (45) and immunoprecipitations (46) were performed as described previously. When indicated, signals on immunoblots were analyzed using the NIH Image program with the appropriate serial dilution standard curves.
Given that MDM2 can be up-regulated by p73, we next examined the expression of MDM2.

**Fig. 3. p73 increases the half-life of MDM2 protein.** H1299 cells (1 × 10⁶ cells/10-cm dish) were transfected with 10 μg of MDM2 plasmid DNA together with either 6 μg of control vector (Lanes 1–4) or 6 μg of p73α plasmid DNA (Lanes 5–8). When the DNA was washed out, each transfection was divided into four 6-cm plates, and the cells were allowed to grow for another 24 h. Cycloheximide (25 μg/ml) was added into the medium, and the cells were harvested for preparation of extracts at the indicated time points. Immunoblotting was performed with anti-MDM2 (Ab-1) and anti-tubulin antibodies. Signals on the immunoblots were analyzed using the NIH Image program, and the MDM2 signals were normalized with the tubulin signals (graph). The average and SDs of three experiments are shown in the graph.

(Lane 5). As we have shown previously (31), coexpression of p73 with MDM2 resulted in the stabilization of p73 (Lanes 3 and 6). A similar effect of p73 on endogenous MDM2 could be seen with the p53-positive U2OS cells (see Fig. 6C). Importantly, the R292H transcriptional inactive mutant of p73α caused a substantially smaller increase in MDM2 (Fig. 6C), suggesting that transcriptional activity of p73 was required for the increase in MDM2.

We next investigated whether p73 could affect MDM2 on the posttranslational level in addition to acting on the level of MDM2 transcription. MDM2 plasmids were cotransfected with either control plasmids or p73 plasmids into H1299 cells. At 24 h after transfection, cycloheximide was added to block the overall protein synthesis in the cells, and the stability of MDM2 was analyzed by immunoblotting as described previously (31). Fig. 3 shows that MDM2 protein was significantly more stable in cells that were cotransfected with p73 plasmids than in cells transfected with MDM2 plasmids alone. These data do not necessarily imply that a direct interaction between p73 and MDM2 was responsible for the stabilization of MDM2. It is conceivable that cell cycle arrest or other effects caused by p73 have an indirect effect on the MDM2 stability. Taken together, these data indicate that expression of p73 could lead to an increase in MDM2, possibly due to increases in both the transcription and protein stability of MDM2.

**Decrease in p53 Level by Cooperation between MDM2 and p73.** Given that MDM2 can be up-regulated by p73, we next examined the potential effects of p73-induced MDM2 on p53. All three proteins, p53, p73, and MDM2, were expressed in the p53-null H1299 cells. As expected, expression of MDM2 resulted in the reduction of p53 protein in a dose-dependent manner (Fig. 4, A and B, Lanes 1–3). Significantly, in the presence of p73α or p73β, p53 expression was further reduced (Lanes 4 and 5). A similar amount of total cell lysates was loaded in each lane as indicated by immunoblotting for tubulin.

Interestingly, the level of transfected MDM2 was not significantly increased by the presence of p73 (although the amount of endogenous MDM2 was increased by p73; see Fig. 2). This suggests that the destabilization of p53 by p73 could be due to a mechanism other than the transfected MDM2.

**Enhancement of MDM2-mediated Degradation of p53 by p73.** We next investigated whether the p53 induced after DNA damage was also affected by the presence or absence of p73. In p53-positive U2OS cells, treatment with Adriamycin (a topoisomerase II inhibitor) induced the expression of p53 (Fig. 5A, Lanes 1 and 3). Strikingly, expression of p73 in these cells reduced the level of p53 in untreated cells (Lanes 1 and 2). Moreover, expression of p73 also abolished the induction of p53 by Adriamycin (Lane 4). Similarly, we found that p53 could be induced in U2OS cells by UV in a time-dependent manner (Fig. 5B). The induction of p53 by UV was delayed when p73 was expressed in the cells (Lanes 3 and 4). A more profound inhibition of p53 responses to Adriamycin and UV by p73 was seen in the human mammary adenocarcinoma MCF-7 cells (Fig. 5, C and D). The levels of tubulin or topoisomerase IIα were used to indicate similar loading of total cell lysates in the above-mentioned experiments.

To see whether expression of p73 affected the association between p53 and MDM2, the amount of p53-MDM2 complexes was determined by immunoblotting the MDM2 immunoprecipitates with anti-p53 antibodies. Fig. 6A shows that coexpression of p73 decreased the amount of p53 found in the MDM2 immunoprecipitates (Lanes 1 and 2). The same results were obtained with endogenous p53 and MDM2 in U2OS cells in the absence (Fig. 6A, Lanes 3 and 4) or presence (Fig. 6B) of DNA damage. In these U2OS cells, endogenous MDM2 was strongly induced when p73 was expressed (Fig. 6C, Lane 2). A substantially lower induction of MDM2 protein was seen when the transcriptional inactive mutant of p73 (R292H) was expressed (Lane 3). Hence the decrease in MDM2:p53 complexes occurred when the MDM2 level was increased. One possibility is that the MDM2 that was induced by p73 expression could bind to p53 and target p53 for degradation. This resulted in a reduction of the total amount of p53 and of the p53 that associated with MDM2 at the end of the experiment.

To test the above-mentioned hypothesis, we used the proteasome inhibitor LLnL to block the degradation of p53. Fig. 6D shows that...
Fig. 5. p73 reduces DNA damage-dependent p53 expression. A. U2OS cells were cotransfected with plasmas expressing p73α and cell surface marker CD2. At 24 h after transfection, the cells were treated with 0.4 μg/ml Adriamycin for 6 h. The cells containing CD2 marker were then separated by magnetic bead selection, and cell extracts were prepared. The levels of p53, topoisomerase IIα, and tubulin were determined by immunoblotting. B. U2OS cells were transfected with p73α or control vector. At 24 h after transfection, the cells were exposed to 30 J/m2 UV and harvested for extract preparation at the indicated time points. The levels of endogenous p53 and topoisomerase IIα were determined by immunoblotting. C. MCF-7 cells were transfected with p73α or control vector. At 24 h after transfection, the cells were treated with 0.4 μg/ml Adriamycin and harvested for extract preparation at the indicated time points. The levels of endogenous p53 and tubulin were determined by immunoblotting. The transcribed HA-tagged p73α was detected by immunoblotting with 12CA5. HA-tagged p73α migrated very close to the 12CA5-cross-reactive bands in these cell extracts (indicated by the asterisks). D, same as B, except that MCF-7 cells were used.

DISCUSSION

In this study, we show that expression of p73 can lead to a decrease in the level of p53. The reduction of p53 is likely to be due to an increase in MDM2 level (by transcription and protein stabilization), which in turn can target p53 for degradation.

eexpression of p73 but not R292H mutant decreased the level of p53 that associated with MDM2 (Lanes 2–4). This datum shows that the reduction of p53 level by p73 required the transcriptional activity of p73. Treatment with LLnL increased the amount of p53 associated with MDM2 (Lanes 5–7). Furthermore, a higher ratio of increase of p53 after LLnL treatment was seen in cells expressing p73 in comparison with cells transfected with control vector or p73 (R292H) mutant. We also consistently found that in the presence of LLnL, there was slightly more p53 associated with MDM2 in p73-transfected cells than in cells transfected with control vector or p73 (R292H) mutant. Taken together, these data indicate that expression of p73 can modulate the level of p53. This modulation of p53 is likely to be due to an increase in MDM2 level (by transcription and protein stabilization), which in turn can target p53 for degradation.

It is interesting that MDM2 protein was stabilized in the presence of p73 (Fig. 3). MDM2 is degraded by the ubiquitination proteasome pathway, but there is no report that MDM2 can be stabilized by binding to p73. As discussed above, our data do not necessarily imply that a direct interaction between p73 and MDM2 was responsible for the stabilization of MDM2. It has been shown that binding of MDM2 to other proteins, such as the MDM2-related protein MDMX, can stabilize MDM2 (55, 56).

p73 has been reported to interact with p53 in other respects. Hetero-oligomers between the p53 and p73 family are possible, but they tend to form homo-oligomers (22). In another report, when p73α was expressed in human ovarian carcinoma cell line A2780, endogenous p53 transcriptional activity was markedly decreased (57). Vikhanskaya et al. (57) attributed this to a possible sequestration of
p53 from its DNA binding site through competitive binding with p73. Conversely, it is conceivable that the expression level of p53 could also affect the level and activity of p73. In this connection, it has been shown that some tumor-derived mutants of p53 can inhibit the function of p73 (49). The mechanisms of this inhibition are unknown.

Whether the stabilization of p73 by MDM2 produces more active p73 or more inactive MDM2-p73 complexes is still a contentious issue. MDM2 is able to reduce p73-dependent transcription in some in vitro reporter assays (30, 32, 33), but the growth-inhibitory activity of p73 is increased in the presence of MDM2 (31). It is conceivable that there is a balance between the increase in p73 transcription activity due to an increase in protein stability and the decrease in p73 transcription activity due to binding of MDM2 to the transactivation domain. In support of this argument, we found that a relatively high concentration of MDM2 was required to inhibit p73-dependent transcription of the MDM2 promoter (Fig. 1). It is likely that the effect of MDM2 on p73 may be different with different p73-responsive promoters. For instance, we found that coexpression with MDM2 significantly increased p73 transactivation activity on the p21CIP1/WAF1 promoter.5

An important inference from these data is that we expect that an increase in p73 (rather than a loss of function as in p53) can lead to deregulation of the DNA damage checkpoint. This is due to an indirect effect because elevated levels of p73 can lead to a reduction of active p53. This may explain the fact that very little loss of function of p73 has been seen in cancer and that p73-deficient mice do not develop tumors spontaneously (25). Hence, it would be very interesting to examine whether p73 is overexpressed in any cancer tissues. Another prediction from the hypothesis presented here is that the level of p53 may decrease when p73 is stabilized after the activation of c-Abl.

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