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-Abl (34–36). Interaction between p73 and c-Abl occurs through the SH3 domain of c-Abl 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, pheromonal, 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 epidermal 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, Labège, France). MDM2 in pCMV was a gift from Dr. Bert Vogelstein (Howard Hughes Medical Institute, Johns Hopkins Oncology Center, Baltimore, MD). Human p53 in pRcCMV was a gift from Dr. Arnold Levine (Princeton University, Princeton, NJ). The β-galactosidase construct was a gift from Dr. Yan Chen (Indiana University School of Medicine, IN). CD2 construct was a gift from Dr. Chris Norbury (ICRF, University of Oxford, Oxford, United Kingdom). MDM2 promoter-luciferase reporter construct 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

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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 inhibited by a higher concentration of MDM2. These data indicate that the MDM2 promoter can be transactivated by p73 in a p53-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 Mr 55,000 and the Mr 90,000 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, both the Mr 55,000 and Mr 90,000 forms of endogenous MDM2 were seen in SAOS-2 cells (Lane 2), but only the Mr 90,000 form was present in H1299 cells.

DMEM supplemented with 10% v/v fetal bovine serum 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 transfet cells in 10-cm plates. The total amount of DNA for each transfection was adjusted to the same level using vectors with the same promoter. Cells were grown for an additional 24 h after transfection before harvesting cell extracts. Cell-free extracts were prepared as described previously (43). The protein concentration of cell lysates was measured with the bicinchoninic acid protein assay system (Pierce) using BSA as a standard. Selection of CD2 cell surface marker was performed according to the manufacturer’s instructions (DYNAL, Oslo, Norway).

Luciferase and β-Galactosidase Assays. Luciferase assays and β-galactosidase assays were performed exactly as described previously (44).

Antibodies and Immunological Methods. Monoclonal antibody DO-1 against p53 (sc-126) and rabbit anti-p21\(^{WAF1/CIP1}\) antibodies (sc397) 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.

Fig. 1. MDM2 is a transcriptional target of p73. A, H1299 cells were cotransfected with plasmids expressing the MDM2 promoter-luciferase reporter (0.2 μg) and β-galactosidase (0.5 μg), together with control vector and plasmids expressing p53, p53 and MDM2, or MDM2 (10 μg each), as indicated. Cell extracts were prepared 24 h after transfection, and the luciferase and β-galactosidase activities were determined. The luciferase activities were normalized with the β-galactosidase activities and expressed as a percentage of p53 without MDM2. The average of three independent experiments and their SDs are shown. B, H1299 cells were cotransfected with plasmids expressing the MDM2 promoter-luciferase reporter and β-galactosidase, together with control vector and plasmids expressing p73α, p73α(R292H), and MDM2 (10 μg each), as indicated. The luciferase activities were measured as described in A and expressed as a percentage of p73α. C, H1299 cells were cotransfected with plasmids expressing the MDM2 promoter-luciferase reporter, β-galactosidase, p53 or p73α (10 μg), and an increasing amount of MDM2-expressing plasmids. The luciferase activities were measured as described in A and expressed as a percentage of p53 or p73α without MDM2.
experiments are shown in the protein in a dose-dependent manner (Fig. 4, expected, expression of MDM2 resulted in the reduction of p53, p73, and MDM2, were expressed in the p53-null H1299 cells. As potential effects of p73-induced MDM2 on p53. All three proteins, Given that MDM2 can be up-regulated by p73, we next examined the increase in MDM2 (Fig. 6), suggesting that transcriptional activity of p53 was required for the increase in MDM2. 

C

increase in MDM2 (Fig. 6 B), suggesting that transcriptional activity of 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 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...
expression 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.

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-mediated proteolysis. The MDM2 promoter can be activated by both p73α and p73β (Fig. 1). The transcriptional targets of p73 are similar but not identical to those of p53 (22). The MDM2 promoter can be activated to varying degrees by different isoforms of p73 (29, 33, 47–49), although other studies showed no effect of p73 on MDM2 transcription (50). Furthermore, the activation of the p21CIP1/WAF1 promoter by p73 appears to be different from p53. We think that one of the major reasons that p53 decreased when p73 was expressed is because MDM2 was transactivated by p73, which in turn interacted with p53 and targeted it for degradation.

Conceptually, the MDM2 that is induced by p73 can bind to either p53 or p73. Binding of MDM2 to p53 targets p53 for degradation, but binding of MDM2 to p73 stabilizes p73 (52). In support of this, there is an increase in MDM2-p73 complex formation when MDM2 is coexpressed with p73 (31). The molecular basis that underlies p73 binding to MDM2 without subsequent degradation is unknown. One possibility is that the COOH terminus of p53, which is necessary for efficient MDM2-mediated degradation of p53, is not conserved in p73 (53). Recently, Gu et al. (54) have defined the sequence element unique to p53 that is absent in p73 (residues 92–112 of p53), which is essential for its regulation by MDM2.

In contrast to p73, p53 is rapidly degraded when it interacts with MDM2. Hence, relatively little p53 could be detected in the MDM2 immunoprecipitates unless it is stabilized by the proteasome inhibitor LLnL (Fig. 6). Given that both p53 and p73 could bind to MDM2, it is possible that p53 and p73 could compete for MDM2. This would tend to produce an increasing amount of p73–MDM2 complexes and a decreasing amount of p53–MDM2 complexes. The fact that expression of p73 resulted in a reduction of p53 indicates that MDM2 was available for binding to p53 even when p73 was overexpressed. This could be because the affinity of MDM2 for p53 is higher than that for p73 or because the level of MDM2 is higher than that of p73.

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
transfection, the cells were treated with 0.4 mg/ml Adriamycin for an additional 6 h. Cell extracts were prepared, and 200 μg were immunoprecipitated with anti-MDM2 antibody. The bound p53 was detected by immunoblotting using anti-p53 antibody. Control plasmids or plasmids expressing p73α or p73α(R292H) were transfected into U2OS cells as indicated. Cell extracts were prepared at 24 h after transfection, and endogenous MDM2 was detected by immunoprecipitation (from 200 μg) followed by immunoblotting with anti-MDM2 antibody Ab-1. D, U2OS cells were transfected with control vectors or plasmids expressing p73α or p73α(R292H). At 22 h after transfection, cells were treated with either buffer (Lanes 2–4) or 50 μM LLnL (Lanes 5–7) for 6 h. Cell extracts were prepared, and 200 μg were immunoprecipitated with anti-MDM2 antibody. The bound p53 was detected by immunoblotting using anti-p53 antibody. C, control plasmids or plasmids expressing p73α or p73α(R292H) were transfected into U2OS cells as indicated.

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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