Subcellular Distribution of p53 and p73 Are Differentially Regulated by MDM2

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Abstract

The binding of MDM2 targets p53, but not p73, for degradation, whereas it suppresses the transactivating function of both proteins. MDM2 also mediates p53 nuclear export, but its role in the regulation of p73 distribution is unknown at the present time. We show here that, in sharp contrast to p53, MDM2 induces p73 to form nuclear aggregates that colocalize with MDM2 but are distinct from the promyelocytic leukemia dots. The MDM2 ring-domain that is necessary for mediating p53 nuclear export is not required for the induction of the p73 nuclear aggregates. Using a domain-swapping approach, we demonstrate that the inability of p73 to nuclear-export is attributable to its nonfunctional nuclear-export sequence.

Introduction

p73 is a newly identified member of p53 family. Consistent with the substantial sequence homology shared by the two proteins, p73 carries out some functions attributed to p53, such as the induction of cell cycle arrest and apoptosis (1). An important regulator of p53 activity is MDM2, which binds directly to the NH2 terminus of p53. Binding of MDM2 to p53 not only blocks the TAD4 of p53, inhibiting its ability to function as a transcription factor, but also targets p53 for degradation through proteasome-dependent proteolysis (2). MDM2 also binds to and inhibits p73 transcription activity, but binding of MDM2 fails to target p73 for degradation (1). One of the activities of MDM2 is to function as a mediator of p53 nuclear export; blocking of nuclear export has been shown to abrogate MDM2-mediated degradation of p53 (3–6), suggesting the importance of nuclear export in the regulation of MDM2-mediated p53 degradation. Whereas the NES of MDM2 was shown to play an important role in mediating p53 nuclear export (4), p53 also has a leucine-rich, rev-like NES at its CT, demonstrating that subcellular distribution of p73 and p53 are differentially regulated by MDM2. In contrast to p53 that is nuclear exported upon coexpression of MDM2, p73 accumulates as nuclear aggregates in the MDM2-expressing cells. We further show that the inability of p73 to be nuclear exported is attributable to its nonfunctional NES.

Materials and Methods

Cell Culture, Transfection. H1299 cells, SAOS-2 cells, and U2OS cells (American Type Culture Collection) were maintained in Eagle’s minimal essential medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum (Sigma Chemical Co.). Cells were transfected by a calcium-phosphate method as described (9). Luciferase activity was measured 24 h posttransfection using a Lumat 9507 illuminometer (EG & G Berthold) as described previously (9). The chimera vectors were generated with the method described previously (9). Identities of the constructs were verified by restriction digestion and by DNA sequencing (Harvard Cancer Center, Boston, MA).

Preparation of Whole Cell Extracts and Immunoblot Analysis. Cells were transfected in 60-mm plates with 8 μg of DNA and harvested at 24 h posttransfection. Cells were lysed in 100 μl of lysis buffer [10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1% Triton X-100, 150 mM NaCl, 1 mM DTT, 10% glycerol, 0.2 mM phenylmethylsulfonyl fluoride, and protease inhibitors] by incubating on ice for 30 min, and the extracts were centrifuged at 13,000 rpm for 15 min to remove cell debris. Protein concentrations were determined by the Bio-Rad protein assay (Bio-Rad, Hercules, California). After addition of 5× loading buffer, the samples were incubated at 95°C for 5 min and resolved through SDS-PAGE. Proteins were transferred to nitrocellulose membranes (Schleicher & Schuell) and probed with the indicated antibody. Proteins were visualized with an enhanced chemiluminescence detection system (NEN).

Subcellular Distribution Assay. Cells were grown on Chamber Slides (Nunc, Naperville, IL) and transfected with the indicated vector. Cells were washed with cold PBS 36 h after transfection and fixed with 4% paraformaldehyde (Sigma Chemical Co.) for 30 min at 4°C. After washing with PBS, the cells were permeabilized with ice cold 0.2% Triton X-100 for 5 min. The slides were washed with PBS, blocked with 0.5% BSA in PBS for 30 min, washed with PBS, and then incubated with the indicated primary antibody at 37°C for 1 h. After washing with PBS three times, the slides were incubated with secondary antibody (Texas red-X goat antimouse LGg; Molecular Probes) and DAPI (10 μg/ml; Sigma Chemical Co.) for 1 h. After PBS washing, the slides were mounted with Fluoromount-G (Southern Biotechnology Associates) containing 2.5 mg/ml 4-propyl gallate (Sigma Chemical Co.). Specimens were examined under a fluorescent microscope (Zeiss).

Results

Subcellular Distribution of p53 and p73 Are Differentially Regulated by MDM2. Given the ability of MDM2 to mediate p53 nuclear export and the conserved NES in p73, we asked whether the binding of MDM2 to p73 could also result in redistribution of this p53 analogue into the cytoplasmic compartment. To accomplish this, GFP-tagged p53 or p73 vectors were generated to facilitate analysis of the subcellular distribution. When transfected alone into U2OS cells, both p53 and p73β were localized mainly to the nucleus (Fig. 1A, panels 1 and 5, top). Consistent with its role as a mediator of p53 nuclear export (4–6), coexpression of MDM2 was indeed associated with an increase of cytoplasmically distributed green p53 protein (Fig. 1A, panel 2, top). Surprisingly, however, p73β was found to have only a minor cytoplasmic distribution, whereas the majority of the green p73β were displayed as very bright aggregates confined in the nucleus of MDM2-expressing cells (Fig. 1A, panel 6, top). To determine whether the distinct subcellular relocalization of p53 and p73β was indeed MDM2-dependent, we examined the distribution of MDM2. As expected, MDM2 exhibited an even nuclear distribution when expressed alone (Fig. 1A, panel 7, middle). MDM2 remained in the

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3 The abbreviations used are: TAD, transactivation domain; NES, nuclear export sequence; CT, COOH terminus; DAPI, 4',6-diamidino-2-phenylindole; GFP, green fluorescent protein; PML, promyelocytic leukemia; NLS, nuclear localization sequence; NoLS, nucleo- localization sequence.
nucleus when coexpressed with p53 (Fig. 1A, panel 2, middle), which is consistent with published results (5, 6). Interestingly, cotransfection with p73α/H9251 induced a redistribution of MDM2 into the p73α/H9251 nuclear aggregates (Fig. 1A, panel 6, middle). Almost identical results were obtained with p73α/H9252 (Fig. 1A, panels 3 and 4). DAPI staining was performed to distinguish the nucleus from the cytoplasm (Fig. 1A, bottom panels). To quantify the results, green fluorescence-positive cells were grouped as exclusive/strong nuclear (EN/SN), exclusive/strong cytoplasmic (EC/SC), equally distributed in two compartments (ED), or nuclear aggregates (NA). A total of 200 cells from random fields were scored for each condition. As shown in Fig. 1B, although MDM2 mediated a significant redistribution of nuclear p53 to the cytoplasmic compartment, ~35–40% of p73 localized as nuclear aggregates in MDM2-expressing cells. A similar MDM2-dependent redistribution of p53 or p73 was observed in H1299 (Fig. 1C) cells, indicating that the finding is not cell-type specific. Together, we demonstrated that p73 accumulated in the nucleus to form discrete nuclear aggregates that were colocalized with MDM2 in contrast with p53 that was exported to the cytoplasm upon coexpression of MDM2.

Characterization of the p73 Nuclear Aggregates. It was reported that inhibition of p53 nuclear export is associated with the accumulation of p53 in the nucleus and that p53 and PML partially colocalized (3). It was therefore of interest to determine whether the nuclear-accumulated p73 aggregates also colocalized with PML. When analyzed with an anti-PML antibody, positive staining displayed the typical PML nuclear dots (Fig. 2A, bottom). Whereas the p73 nuclear
aggregates were induced by coexpression of MDM2, the PML dots were abundant regardless of the expression of MDM2 (Fig. 2A, bottom). Moreover, the PML nuclear dots were mostly distinct from the p73 nuclear aggregates, as immune staining revealed no significant colocalization of the PML dots and the p73 aggregates (Fig. 2A).

Because inhibition of nuclear export results in an increase of p53 protein levels (3, 4), we asked how the nuclear-accumulated p73 correlated with its protein levels. As expected, p53 levels were remarkably reduced by coexpression of MDM2 (Fig. 2B, middle panel, Lane 3 versus Lane 4). p73 protein levels, however, were slightly increased in MDM2-expressing cells (Fig. 2B, middle panel, Lane 1 versus Lane 2).

The inhibitory effect of MDM2 on p53 is a combination of its binding to the p53 TAD and destabilization of p53 protein (1). Although capable of inhibiting the p73 transactivation by blocking the TAD (1), the inability of MDM2 to degrade p73 would predict a reduced potency. To test this, a transcriptional assay was conducted with a reporter construct containing the luciferase gene driven by a p53 enhancer from the PG13 element transfected into H1299 cells. Under the condition where the p73 nuclear aggregates were induced, the transcriptional activity of p73 was down-regulated by MDM2 with a potency comparable with its inhibition of p53-dependent transcription activity (Fig. 2C), a finding consistent with the published report (1). This result indicates that, although unable to degrade p73, MDM2 inhibits transcriptional activity of this p53 homologue as potently as it does that of p53.

The Ring-Domain of MDM2 Is Dispensable for the Induction of the p73 Nuclear Aggregates. The ring-domain of MDM2 has been shown to be essential for mediating p53 nuclear export (5, 6); it was therefore of interest to determine whether induction of the p73 nuclear aggregates also required the MDM2 ring-domain. For the purpose of comparison, other MDM2 mutants (Fig. 3A) defective in the p73-binding, cryptic NoLS (9) or NES were included. The binding of MDM2 to p73 seemed to be critical for its induction of p73 nuclear aggregates, as demonstrated by the inability of the MDM2 (DS8 and 68A) mutant deficient in binding to p73 (not shown) to induce the p73 nuclear aggregates formation (Fig. 3B, panel 3). In contrast to p53 nuclear export that requires an intact MDM2 ring-domain (5, 6),
induction of the p73/MDM2 nuclear aggregates is independent of the ring-domain, as evidenced by the fact that cotransfection of the MDM2 ring-domain mutant with p73 was still associated with formation of the nuclear aggregates (Fig. 3B, panel 5). The NES and NoLS of MDM2 also seemed not to be essential; p73 still formed the nuclear aggregates when the NES or NoLS mutant of MDM2 was coexpressed (Fig. 3B, panels 4 and 6). Results of quantitative analysis are shown in Fig. 3C. Together these results demonstrate that, analogous to its regulation of p53 nuclear export, binding of MDM2 seems to be critical for the induction of p73 nuclear aggregates. In contrast to its role in p53 nuclear export, however, the ring-domain of MDM2 is dispensable for the redistribution of p73.

The p73 NES Is Functionally Inactive. The distinct subcellular redistribution of p53 and p73 in MDM2-expressing cells suggests the existence of structural differences between the proteins. Whereas the oligomerization domain of p53 and p73 share 33% identity, the extreme CT (outside of the oligomerization domain) of the two proteins is much less conserved. Inspection of the primary amino acid sequence reveals that the p53 CT contains five lysine residues that are not conserved in p73. In addition, we showed recently that under conditions where p53 is highly ubiquitinated, p73 exhibits a much lower tendency for ubiquitination (10). According to the current model of p53 nuclear export, the p53 NES is inactive when p53 is in tetramer. Ubiquitination of the p53 CT lysine residues by MDM2 results in the revealing of the NES that then permits p53 to be nuclear-exported (5–7). It is therefore possible that the NES of p73 is unable to be activated because of its lack of the corresponding lysine residues available for ubiquitination. To test this possibility, GFP-tagged p53/p73 chimeras with their CTs swapped were prepared (Fig. 4A). We first tested the chimeras for ubiquitination. Western analysis demonstrated that cotransfection of MDM2 resulted in significant ubiquitination of p53. However, p73Bβ was much less ubiquitinated under the same condition (Fig. 4B, Lane 2 versus Lane 4). Interestingly, swapping the p53 CT into the p73 backbone was associated with a gain in the ability of the p73 chimera to be ubiquitinated in an MDM2-dependent manner, as demonstrated by the appearance of an abundant typical ubiquitinated ladder of p73/p53CT in MDM2-expressing cells (Fig. 4B, Lane 4 versus Lane 6). The increased ubiquitination of the p73 chimera, however, could be attributable to either the lysine residues from the swapped p53 CT or to a conformational alteration caused by domain swapping so that lysine residues from region of p73 other than the CT became ubiquitinatable. A p73 chimera harboring a p63 CT that also lacks lysine residues was prepared to test this possibility. In contrast to p73/p53CT, cotransfection of p73/p63CT with MDM2 was not associated with any apparent ubiquitination of this chimera (Fig. 4B, Lane 8), indicating that the increased ubiquitination of the p73/p53CT chimera was indeed transferred by the lysine residues of the p53 CT. If ubiquitination of the lysine residues in the p53 CT could result in revealing the p73 NES, nuclear export of the p73 chimera would be expected when MDM2 is expressed. A GFP-tagged p73/p53CT chimera was generated for assessing its subcellular distribution and a GFP-p73/p63CT chimera was included as a control. However, the p73Bβ/p53CT chimera remained accumulated in the nucleus of MDM2-expressing cells. Similar results were obtained with the p73Bβ/p63CT chimera. Quantitative data analysis are shown in Fig. 4C. Together, these results indicate that the difference in the CT ubiquitination does not contribute to the observed differential subcellular redistribution between p53 and p73.

Although the NES of p53 is conserved in p73, the inability of p73 to be nuclear-exported would suggest a functional incompetence of its NES. We used p53 as a reporter protein to test this possibility. If the p73 NES were functionally defective, swapping the p73 NES into p53 would result in a p53 chimera incapable of nuclear export. The NES swapping chimera was then generated (Fig. 4D, top) to test this possibility. In contrast to wild-type p53 that was nuclear-exported upon coexpression of MDM2 (Fig. 4D, panel 2), p53/p73NES failed to exhibit significant nuclear export. Instead the p53 chimeric protein accumulated in the nucleus and colocalized with MDM2 (Fig. 4D, panel 3). The p73 NES is functionally inactive.
Discussion

Appropriate subcellular localization is crucial for control of p53 function. MDM2, as an ubiquitin E3 ligase (11), binds to p53 and promotes its ubiquitination and the subsequent nuclear export and degradation of p53. MDM2 also binds to p73, but this binding is not associated with significant degradation of p73, suggesting that the protein stability of p53 and p73 are regulated through different mechanisms. In sharp contrast to p53 that is nuclear-exported upon expression of MDM2, p73 was found to accumulate in the nucleus as aggregates in MDM2-expressing cells, suggesting the presence of a structural difference that determines the distinct subcellular redistribution. Comparison of the amino acid sequences of p53 and p73 reveals that the most pronounced difference between the two proteins is that the CT lysine residues are absent in p73. According to the current model of MDM2-mediated p53 nuclear export (5, 6), MDM2-dependent ubiquitination of the CT lysine residues reveals the otherwise buried p53 NES that then permits p53 to be nuclear-exported. It is therefore conceivable that the inability of MDM2 to promote nuclear export of p73 was attributable to the absence of ubiquitinnable lysine residues at the p73 CT. This notion, however, is inconsistent with the finding that the gain of ability to be ubiquitinated in p73/p53CT was not associated with its nuclear export. Although essential to p53 nuclear export, the MDM2 ring-domain, where the E3 ligase activity resides, is dispensable for the induction of p73 nuclear aggregates. This result suggests that ubiquitination plays little role in the control of subcellular redistribution of p73. It has been demonstrated that the ability of p53 to nuclear-export relies on its intrinsic NES (7). Although the p53 NES is conserved in p73, we used the NES swapping chimera to demonstrate that the p73 NES is not functional, thus providing a molecular basis for the failure of p73 to be nuclear-exported in the MDM2-expressing cells.

MDM2 binds to both p53 and p73, but this binding results in completely different subcellular redistributions. Moreover, only p53, and not p73, is targeted for degradation by MDM2. Whether this distinct subcellular distribution contributes to the differing susceptibility to MDM2-mediated degradation remains to be determined. A related question is whether nuclear export is required for degradation of p53 or its homologues. Studies are ongoing to address these issues. Nevertheless, the results presented here demonstrate that related p53 family members take divergent pathways in MDM2-dependent regulation.

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