Mdm2-mediated NEDD8 Modification of TAp73 Regulates Its Transactivation Function

Received for publication, April 17, 2006, and in revised form, August 14, 2006 Published, JBC Papers in Press, September 14, 2006, DOI 10.1074/jbc.M603654200

Ian R. Watson†‡, Alvaro Blanch†, Dan C. C. Lin†, Michael Ohh§¶, and Meredith S. Irwin*†‡

From the †Cancer Research Program and Division of Haematology-Oncology, Hospital for Sick Children, Toronto, Ontario MSG 1X8, Canada and the ‡Department of Laboratory Medicine and Pathobiology, §Department of Paediatrics and Institute of Medical Science, University of Toronto, Toronto, Ontario M5S 1A8, Canada

Mutations in p73 are rare in cancer. Emerging evidence suggests that the relative expression of various p73 isoforms may contribute to tumorigenesis. Alternative promoters and N-terminal splicing result in the transcription and processing of either full-length (TA) or N-terminally truncated (∆N) p73 isoforms. TAp73 possesses pro-apoptotic functions, while ∆Np73 has anti-apoptotic properties via functional inhibition of TAp73 and p53. Here, we report that TAp73, but not ∆Np73, is covalently modified by NEDD8 under physiologic conditions in an Mdm2-dependent manner. Co-expression of NEDP1, a cysteine protease that specifically cleaves NEDD8 conjugates, was shown to deeddylate TAp73. In addition, blockade of the endogenous NEDD8 pathway increased TAp73-mediated transactivation of p53- and p73-responsive promoter-driven reporter activity, and in conjunction, neddylated TAp73 species were found preferentially in the cytoplasm. These results suggest that Mdm2 attenuates TAp73 transactivation function, at least in part, by promoting NEDD8-dependent TAp73 cytoplasmic localization and provide the first evidence of a covalent post-translational modification exclusively targeting the TA isoforms of p73.

p73 is a member of the p53 family that binds p53 DNA-binding sites, transactivates p53-target genes, and induces apoptosis (1–3). p73 mutations are rarely observed in cancer (4). However, p73 exists as multiple C-terminal (α, β, γ, δ, ε, and η) and N-terminal (TA and ∆N) isoforms, and accumulating evidence suggests that the relative expression and stability of the different N-terminal isoforms of p73 may play a role in tumorigenesis. Full-length TAp73 isoforms have pro-apoptotic properties, whereas the N-terminally truncated ∆Np73 isoforms, which lack the transactivation domain due to the use of a promoter within intron 3 and alternative N-terminal mRNA splicing, have anti-apoptotic properties. ∆Np73 acts as a negative inhibitor of both TAp73 and p53, either via hetero-oligomerization or through competition for DNA-binding sites (5–10). Furthermore, ∆Np73 expression has been shown to be elevated in a number of human cancers, including breast, ovarian, hepatocellular, prostate, colon, and neuroblastoma tumors (11–15). Increased ∆Np73 expression has been associated with poor prognosis in patients, and this finding has been attributed to ∆Np73 inhibition of p53 and TAp73 causing chemoresistance (10, 12, 16, 17). Conversely, a recent study demonstrated that aged p73 heterozygous mice develop spontaneous tumors, and in comparison with p53 heterozygous mice, mice heterozygous for both p53 and p73 have different tumor spectrums and higher tumor burden and incidence of metastasis, presumably due to the loss of function of the pro-apoptotic TAp73 isoforms (18). Studies have also established a role for TAp73 in chemotherapy-induced apoptosis and the status of TAp73 may be an important determinant of chemotherapeutic efficacy in humans (17, 19, 20). Therefore, therapeutic modulation of the relative levels of TAp73 and ∆Np73 isoforms has potential therapeutic benefits in treating human cancers.

The RING finger E3 ligase Mdm2 is one of the major regulators of p53. Mdm2 promotes ubiquitination of p53 on multiple C-terminal lysines resulting in its degradation via the 26S proteasome (21–24). NEDD8 is an ubiquitin-like modifier (UBL) most similar to ubiquitin. As such, NEDD8 conjugation to its substrates is mediated by an analogous pathway involving concerted actions of E1 (activating), E2 (conjugating), and E3 (ligating) enzymes (25). Ultimately, the mature NEDD8 with exposed C-terminal glycine forms an isopeptide bond with a lysyl residue of receptive substrates (26). Interestingly, Mdm2 was shown recently to regulate NEDD8 conjugation of p53 inhibiting its transcriptional activity and thereby revealing another mechanism of p53 regulation via Mdm2 at a post-translational level (27).

Many of the same post-translational modifications that affect p53 function, such as phosphorylation, acetylation, and sumoylation, and their respective regulators Chk1, p300/CBP, and PIAS1, regulate p73 (28–30). In addition, specific regulators of p73 have been identified, such as the NEDD4-like E3 ubiquitin ligases NEDL2 and Itch (31, 32). The role of Mdm2 in the regulation of TAp73 is complex but also less well understood. For example, Mdm2 does not promote the degradation of TAp73...
but rather stabilizes TAp73 (33–35). The mechanism underlying Mdm2-mediated stabilization of TAp73 is unknown. TAp73 contains a functional nuclear export signal and Mdm2 was shown to influence the subcellular redistribution of TAp73 (36–39). Moreover, Mdm2 inhibits both TAp73-mediated transactivation and apoptosis (33, 35, 40). In support, Mdm2 was shown to repress the transcriptional function of TAp73 by interrupting its association with transcriptional co-activators p300/CBP (35). However, the molecular mechanisms associated with the various aforementioned properties of Mdm2-mediated regulation of p73 remain unclear, and post-translational modifications that differentially regulate the TA and ΔN isoforms of p73 have yet been elucidated.

Here, we show that TAp73 is covalently modified by NEDD8 in an Mdm2-dependent manner. The ΔNp73 isoform, which does not possess the N-terminal Mdm2-binding site, failed to be modified by NEDD8 and the cysteine protease NEDP1, which specifically cleaves neddylated substrates, promoted the deneddylation of TAp73. Importantly, an intact NEDD8 pathway attenuated TAp73 transcriptional activity in ts41 Chinese hamster ovary (CHO) cells, and Mdm2 promoted preferential cytoplasmic localization of NEDD8-TAp73. Taken together, our findings provide a mechanistic insight into Mdm2-mediated NEDD8-dependent regulation of TAp73. In addition, to our knowledge, this is the first evidence of p73 regulation via covalent post-translational modification exclusively targeting the TAp73 isoform.

MATERIALS AND METHODS

Cells—HEK293 human embryonic kidney and SAOS-2 cells were obtained from American Type Culture Collection (Rockville, MD) and maintained in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (Hyclone, Logan, UT) at 37 °C in a humidified 5% CO2 atmosphere. The ts41 and wild type CHO cell lines were maintained essentially as described previously (41).

Antibodies—Monoclonal anti-hemagglutinin (HA) (12CA5) and polyclonal anti-HA (Y11) antibodies were obtained from Roche Applied Science and Santa Cruz Biotechnology (Santa Cruz, CA), respectively. Monoclonal anti-T7 antibody was obtained from Novagen (Madison, WI). Polyclonal anti-NEDD8 antibody was obtained from Alexis Biochemicals (Lansen, Switzerland). Monoclonal anti-p73 (ER15) antibody was described previously (3). Monoclonal anti-p73β (GC15) antibody was obtained from Oncogene Research (La Jolla, CA). Monoclonal anti-α-tubulin antibody was obtained from Sigma. Monoclonal anti-heterogeneous nuclear ribonucleoprotein antibody was obtained from Abcam (Cambridge, MA). Polyclonal anti-p73 (2301) antibodies were raised against a glutathione S-transferase fusion of the α C terminus of murine p73. Rabbits were immunized and boosted at 6-week intervals using standard methods, and antibodies were subsequently affinity-purified.

Plasmids—pcDNA3-T7-NEDD8 and pcDNA3-T7-NEDD8/ΔGG were described previously (42). The pCMV-Hdm2 plasmid was generously provided by Dr. Samuel Benchimol. The pcDNA3-HA-TAp73β and pcDNA3-myc-ΔNp73β plasmids were described previously and generously provided by Dr. Gerry Melino (43) and Dr. Freda Miller, respectively (7). The pcDNA3.1/V5-His-NEDP1 and pcDNA3.1/V5-His-NEDP1(C163A) plasmids were gifts from Dr. Ronald T. Hay (44). pcDNA3-myc-APP-BP1 was generously provided by Dr. Dimitris P. Xirodimas (27). pcDNA3-p53 and PG-13-luciferase reporter constructs were described previously (45). The PG-13-luciferase reporter plasmid that contains 13 contiguous p53 DNA-binding sites upstream of the luciferase gene and the MG-15-luciferase reporter plasmid that contains mutated p53 DNA-binding sites upstream of the luciferase gene were kindly provided by Dr. Wafik El-Deiry.

Immunoprecipitation and Immunoblotting—Cells growing in monolayers were removed from 10 cm tissue culture plates by scraping and collected by centrifugation. The cells were washed once with cold phosphate-buffered saline, resuspended in 10% phosphate-buffered saline, and lysed in 90% lysis buffer (pH 7.35) (20 mM Tris, 250 mM NaCl, 3 mM EDTA, 3 mM EGTA, 0.5% Nonidet P-40, 2 mM dithiothreitol, 5 mM N-ethylmaleimide, 2 mM iodoacetamide) containing 1% SDS and supplemented with complete protease inhibitors (Roche Diagnostics). The samples were incubated at 100 °C for 20 min, followed by centrifugation at 13,200 rpm for 10 min to remove cell debris. Protein concentrations were determined by Bradford method and whole cell lysates were diluted 10 times with lysis buffer without SDS in preparation for the immunoprecipitation procedure. Immunoprecipitation and immunoblotting procedures were performed as described previously (3). Briefly, immunoprecipitations were carried out with the indicated antibody for 2 h at 4 °C with protein A-Sepharose (Amersham Biosciences). Immunoprecipitates were washed five times with NETN buffer (2 mM Tris (pH 8.0), 5 mM NaCl, 0.5 mM EDTA (pH 8.0), 0.5% Nonidet P-40) eluted by boiling in SDS-containing buffer, and separated by SDS-PAGE. Proteins were transferred to nitrocellulose membranes (Bio–Rad) for Western analysis.

Subcellular Fractionation—Cells were trypsinized, washed twice with cold phosphate-buffered saline, and pelleted by centrifugation at 1000 rpm at 4 °C for 3 min. Cells were resuspended in 250 μl of lysis buffer A (10 mM HEPES (pH 7.9), 10 mM KCl, 1.5 mM MgCl2, 0.34 mM sucrose, 10% glycerol, 1 mM dithiothreitol) containing 0.1% Triton X-100 and supplemented with complete protease inhibitors (Roche Diagnostics). Following a 7-min incubation on ice, the nuclear fraction was pelleted by centrifugation at 3600 rpm for 5 min at 4 °C, and the cytoplasmic cell fraction was decanted. The nuclear pellet was washed once with buffer A without Triton X-100. The nuclear pellet was resuspended in 100 μl of lysis buffer B (0.2 mM EGTA (pH 8), 3 mM EDTA (pH 8), 1 mM dithiothreitol) and incubated on ice for 30 min while undergoing periodic vortexing. The nuclear fraction was collected following centrifugation at 4000 rpm for 5 min at 4 °C. Protein concentrations were determined by Bradford method, and immunoprecipitations were performed as described above.

Dual Luciferase Reporter Assay—The ts41 and wild type CHO cells were transiently transfected using FuGENE 6 (Roche Diagnostics) with 20 ng of pcDNA3-HA-TAp73β or pcDNA3-p53, 20 ng of either PG-13- or p21-luciferase reporter construct, and 0.1 ng of Renilla luciferase construct. In cases where APP-BP1 and TAp73β were co-expressed in the ts41
Mdm2 Mediates TAp73 Neddylation
cells, 10 ng of pcDNA3-HA-TAp73β was co-transfected with either 50, 100, or 150 ng of pcDNA3-myc-APP-BP1. Following transfection, cells were incubated overnight at 33 °C and were maintained either at 33 °C or shifted to 39 °C for an additional 24 h. SAOS-2 cells were similarly transfected with 20 ng of pcDNA3-HA-TAp73α or HA-TAp73β in combination with 80 ng of pcMV-Hdm2 and 100 ng of pcDNA3-T7-NEDD8 where indicated, followed by an incubation period of 48 h at 37 °C. The total amount of transfected DNA was equilibrated to 200 ng using an empty pcDNA3 plasmid. Luciferase and Renilla activities were measured using Dual Luciferase Reporter Assay System (Promega, Madison, WI) according to the manufacturer's instructions on a Lumat LB 9507 luminometer (Berthold Technologies).

RESULTS AND DISCUSSION
TAp73β is Modified by NEDD8 via Mdm2—Mdm2 promotes NEDD8 conjugation of p53 to negatively regulate its transcriptional activity (27). We asked whether p73 is subjected to a similar NEDD8-dependent regulation mediated by Mdm2. HEK293 cells were transiently transfected with plasmids encoding HA-TAp73β in combination with plasmids encoding T7-NEDD8 and human Mdm2 (Hdm2). Cells were lysed, immunoprecipitated with anti-HA antibody, bound proteins resolved on SDS-PAGE, and visualized by immunoblotting with anti-HA (Fig. 1A, left panel) and anti-T7 (right panel) antibodies. Slower co-migrating HA-TAp73β proteins were readily detectable in cells transfected with plasmids encoding HA-TAp73β and T7-NEDD8, and Hdm2 (Fig. 1A, compare lanes 4 and 9). These modified HA-TAp73β proteins were absent in cells transfected with plasmids encoding HA-TAp73β alone (Fig. 1A, lanes 2 and 7). As expected, co-expression of Hdm2 and T7-NEDD8 in the absence of HA-TAp73β did not generate modified HA-TAp73β proteins (Fig. 1A, lanes 5 and 10). Therefore, the slower migrating forms of TAp73β contain both HA and T7 epitopes, suggesting that T7-NEDD8 covalently conjugates to TAp73β. Notably, in the absence of exogenous Hdm2, ectopic co-expression of HA-TAp73β and T7-NEDD8 generated similar slower co-migrating HA-TAp73β (Fig. 1A, compare lanes 8 and 9 under dark exposure). In a reciprocal experiment, anti-T7 immunoprecipitation was performed followed by anti-HA Western analysis (Fig. 1B, right panel). Again, slower migrating TAp73β species containing both HA and T7 epitopes were observed, supporting the notion that HA-TAp73β is modified by T7-NEDD8 (Fig. 1B, lanes 4 and 9). Similar observations were made in the context of HA-TAp73α (data not shown).

To further confirm that the modified TAp73β is conjugated by NEDD8, HEK293 cells were transiently transfected with plasmids encoding HA-TAp73β and T7-NEDD8 and Hdm2. Cells were then lysed, immunoprecipitated with anti-HA antibody, bound proteins separated on SDS-PAGE, and visualized by immunoblotting with anti-HA (Fig. 1C, left panel) and anti-NEDD8 (right panel) antibodies. Similar slower migrating HA-TAp73β species containing both HA and NEDD8 epitopes were observed (Fig. 1C, lanes 7 and 14; compare with Fig. 1A). Taken together, these results strongly suggest that Mdm2 promotes TAp73β covalent modification by NEDD8.

NEDP1 Deneddylates Modified TAp73β—NEDP1 is a cysteine protease that specifically cleaves NEDD8 conjugates on cullins, which are the scaffold components of SCF E3 ubiquitin ligase complexes (44). In addition, NEDP1, but not other UBL deconjugating enzymes (e.g. SUMO-deconjugating enzyme SSP3), was shown to specifically deneddylate NEDD8-modified p53 (27). In a complementary experiment, we asked whether the physiologically relevant NEDD8-deconjugating enzyme NEDP1 could curtail NEDD8-modification of TAp73β. HEK293 cells were transfected with plasmids encoding HA-TAp73β, Hdm2, T7-NEDD8 or con-
NEDDylation of p73 Occurs under Physiological Conditions—Mdm2 binds to the hydrophobic stretch (16QPTFSDY-WKLLP27) within the N terminus of p53. In particular, Phe19, Trp23, and Leu26 residues make direct contact with and are indispensable for binding to Mdm2 (46, 47). These critical Mdm2-binding residues are conserved in the N terminus of TA isoforms of p73 (2). Therefore, we asked whether the ΔNp73β that lacks the Mdm2-binding motif is capable of being modified by NEDD8. HEK293 cells were transiently transfected with plasmids encoding TAp73β or ΔNp73β in combination with T7-NEDD8 and Hdm2. Cells were lysed, immunoprecipitated with either polyclonal T7-NEDD8 and Hdm2, and Hdm2. The cells were lysed and immunoprecipitated (IP) with an anti-p73 (ER15) antibody. Bound proteins were resolved by SDS-PAGE and immunoblotted with an anti-p73 (2301) antibody or an irrelevant isotype-matched control antibody. Bound proteins were resolved by SDS-PAGE and immunoblotted with an anti-p73 (GC15) (left panel) or anti-NEDD8 (right panel) antibody.

Endogenous p73 is modified by NEDD8. HEK293 cells were lysed under denaturing conditions and immunoprecipitated with either polyclonal anti-p73 (2301) or an irrelevant control antibody. Bound proteins were resolved by SDS-PAGE and immunoblotted with an anti-p73 (2301) (left panel) or an anti-NEDD8 (right panel) antibody. As expected, multiple endogenous p73 proteins were observed, indicating the presence of various N- and C-terminal p73 isoforms (Fig. 4, lane 1). Importantly, a co-migrating protein containing both p73- and NEDD8-specific epitopes was observed (Fig. 4, compare lanes 1 and 3). Similar results were observed using an anti-p73 antibody generated against the N terminus of TAp73 (H-79, Santa Cruz Biotechnology) (supplemental Fig. 1), suggesting that, in agreement with the above overexpression data, the modified forms of p73 are most likely the full-length TA isoforms. In addition, a reciprocal immunoprecipitation using anti-NEDD8 antibody followed by an anti-p73 immunoblot showed a single protein species exhibiting both NEDD8 and p73 epitopes (data not shown). Under physiologic conditions, unlike in

Mdm2 Mediates TAp73 Neddylation

NEDDylation of p73 Occurs under Physiological Conditions—Mdm2 binds to the hydrophobic stretch (16QPTFSDY-WKLLP27) within the N terminus of p53. In particular, Phe19, Trp23, and Leu26 residues make direct contact with and are indispensable for binding to Mdm2 (46, 47). These critical Mdm2-binding residues are conserved in the N terminus of TA isoforms of p73 (2). Therefore, we asked whether the ΔNp73β that lacks the Mdm2-binding motif is capable of being modified by NEDD8. HEK293 cells were transiently transfected with plasmids encoding TAp73β or ΔNp73β in combination with T7-NEDD8 and Hdm2. Cells were lysed, immunoprecipitated with either polyclonal T7-NEDD8 and Hdm2, and Hdm2. The cells were lysed and immunoprecipitated (IP) with an anti-p73 (ER15) antibody. Bound proteins were resolved by SDS-PAGE and immunoblotted with an anti-p73 (2301) antibody or an irrelevant isotype-matched control antibody. Bound proteins were resolved by SDS-PAGE and immunoblotted with an anti-p73 (GC15) (left panel) or anti-NEDD8 (right panel) antibody.

Endogenous p73 is modified by NEDD8. HEK293 cells were lysed under denaturing conditions and immunoprecipitated with either polyclonal anti-p73 (2301) or an irrelevant control antibody. Bound proteins were resolved by SDS-PAGE and immunoblotted with an anti-p73 (2301) (left panel) or an anti-NEDD8 (right panel) antibody. As expected, multiple endogenous p73 proteins were observed, indicating the presence of various N- and C-terminal p73 isoforms (Fig. 4, lane 1). Importantly, a co-migrating protein containing both p73- and NEDD8-specific epitopes was observed (Fig. 4, compare lanes 1 and 3). Similar results were observed using an anti-p73 antibody generated against the N terminus of TAp73 (H-79, Santa Cruz Biotechnology) (supplemental Fig. 1), suggesting that, in agreement with the above overexpression data, the modified forms of p73 are most likely the full-length TA isoforms. In addition, a reciprocal immunoprecipitation using anti-NEDD8 antibody followed by an anti-p73 immunoblot showed a single protein species exhibiting both NEDD8 and p73 epitopes (data not shown). Under physiologic conditions, unlike in
Mdm2 Mediates TAp73 Neddylation

The Neddylation Pathway Attenuates TAp73 Transcriptional Activity—Our results show that Mdm2 promotes NEDD8 modification of TAp73. To address the functional significance of TAp73 neddylation, we used a well-characterized ts41 CHO cell line with a temperature-sensitive mutation in the SMC gene (the hamster homologue of human APP-BP1), a component of the E1 NEDD8-activating enzyme, to determine whether an intact NEDD8 pathway affects TAp73 transcriptional activity (41). The ts41 CHO cells were transiently transfected with either p53 or HA-TAp73β with a PG-13-luciferase reporter construct (Fig. 5A) or a p53-binding site upstream of the luciferase gene (data not shown). Recently, Mdm2-mediated neddylation of p53 was shown to inhibit its transcriptional activity (27), and thus, as expected, a similar increase in the PG-13-luciferase reporter activity was observed with p53 upon inactivation of the NEDD8 pathway (Fig. 6A). Furthermore, we did not observe a change in the transcriptional activity of HA-TAp73β in the wild-type CHO cells between 33 °C and 39 °C (Fig. 5B) demonstrating that the increase in TAp73β transcriptional activity in ts41 cells was not attributable to the temperature change. In complementary experiments, a reconstitution of wild type APP-BP1 in ts41 cells inhibited HA-TAp73β transcriptional activity in a dose-dependent manner at the non-permissive temperature (Fig. 5C, and 5D). These results strongly suggest that an intact NEDD8 pathway attenuates TAp73 transcriptional activity.

NEDD8 Modification of TAp73β Promotes Cytoplasmic Localization—We next examined the subcellular localization of neddylated TAp73β as a potential mechanism by which neddylation of TAp73β inhibits its transactivation property. HEK293 cells were transiently transfected with plasmids encoding HA-TAp73β in combination with Hdm2 and T7-NEDD8 or T7-NEDD8ΔGG. As mentioned previously, Mdm2 stabilizes TAp73 via an unknown mechanism (34, 35). Therefore, cells overexpressing HA-TAp73β alone were transfected with higher amounts of plasmid encoding HA-TAp73β as compared with those transfected with plasmids encoding HA-TAp73β and Hdm2 to achieve comparable levels of HA-TAp73β protein. The isolated cytoplasmic and nuclear lysates were immunoprecipitated and immunoblotted with an anti-HA antibody, as well as anti-α-tubulin and anti-heterogeneous nuclear ribonucleoprotein antibodies for validation of fractionation efficiency (Fig. 6A). Expression of HA-TAp73β alone or in combination with Hdm2 and conjugation-defective T7-NEDD8ΔGG resulted in the absence of neddylated HA-TAp73β (lanes 1, 5, and 7). Importantly, in the presence of Hdm2 and T7-NEDD8,

The Neddylation Pathway Attenuates TAp73 Transcriptional Activity—Our results show that Mdm2 promotes NEDD8 modification of TAp73. To address the functional significance of TAp73 neddylation, we used a well-characterized ts41 CHO cell line with a temperature-sensitive mutation in the SMC gene (the hamster homologue of human APP-BP1), a component of the E1 NEDD8-activating enzyme, to determine whether an intact NEDD8 pathway affects TAp73 transcriptional activity (41). The ts41 CHO cells were transiently transfected with either p53 or HA-TAp73β with a PG-13-luciferase reporter construct (Fig. 5A) or a p53-binding site upstream of the luciferase gene (data not shown). Recently, Mdm2-mediated neddylation of p53 was shown to inhibit its transcriptional activity (27), and thus, as expected, a similar increase in the PG-13-luciferase reporter activity was observed with p53 upon inactivation of the NEDD8 pathway (Fig. 6A). Furthermore, we did not observe a change in the transcriptional activity of HA-TAp73β in the wild-type CHO cells between 33 °C and 39 °C (Fig. 5B) demonstrating that the increase in TAp73β transcriptional activity in ts41 cells was not attributable to the temperature change. In complementary experiments, a reconstitution of wild type APP-BP1 in ts41 cells inhibited HA-TAp73β transcriptional activity in a dose-dependent manner at the non-permissive temperature (Fig. 5C, and 5D). These results strongly suggest that an intact NEDD8 pathway attenuates TAp73 transcriptional activity.

NEDD8 Modification of TAp73β Promotes Cytoplasmic Localization—We next examined the subcellular localization of neddylated TAp73β as a potential mechanism by which neddylation of TAp73β inhibits its transactivation property. HEK293 cells were transiently transfected with plasmids encoding HA-TAp73β in combination with Hdm2 and T7-NEDD8 or T7-NEDD8ΔGG. As mentioned previously, Mdm2 stabilizes TAp73 via an unknown mechanism (34, 35). Therefore, cells overexpressing HA-TAp73β alone were transfected with higher amounts of plasmid encoding HA-TAp73β as compared with those transfected with plasmids encoding HA-TAp73β and Hdm2 to achieve comparable levels of HA-TAp73β protein. The isolated cytoplasmic and nuclear lysates were immunoprecipitated and immunoblotted with an anti-HA antibody, as well as anti-α-tubulin and anti-heterogeneous nuclear ribonucleoprotein antibodies for validation of fractionation efficiency (Fig. 6A). Expression of HA-TAp73β alone or in combination with Hdm2 and conjugation-defective T7-NEDD8ΔGG resulted in the absence of neddylated HA-TAp73β (lanes 1, 5, and 7). Importantly, in the presence of Hdm2 and T7-NEDD8,
Mdm2 Mediates TAp73 Neddylation

T7-NEDD8-modified HA-TAp73β was detected and preferentially localized in the cytoplasm (Fig. 6A, lane 3). The level of unmodified HA-TAp73β was also frequently higher in the cytoplasmic fraction. In addition, where the cytoplasmic ectopic expression level of HA-TAp73β was comparable, neddylated HA-TAp73β was not detected in cells transfected with plasmids encoding Hdm2 and T7-NEDD8ΔGG (Fig. 6B). These results suggest that Mdm2 may exert its inhibitory effect on TAp73β transcriptional activity, at least in part, by promoting TAp73β cytoplasmic localization via NEDD8 conjugation of TAp73β, and argue against the notion that the neddylation of HA-TAp73β is simply due to the higher expression level of HA-TAp73β in the cytoplasm. Nevertheless, we have also observed increased accumulation of TAp73β in the presence of ectopic Hdm2, and thus, it is formally possible that Hdm2-mediated accumulation of TAp73β has a role in subcellular trafficking. Previous studies have demonstrated some effects of Mdm2 overexpression on TAp73 subcellular localization. For example, Mdm2 promoted TAp73α redistribution from the nucleus to the paranuclear regions, and upon expression of the Mdm2-related protein MdmX, TAp73α was found primarily in the cytoplasm (39). In addition, TAP73β redistribution to the cytosol and nuclear aggregation was observed in the setting of ectopic Mdm2 expression (36). However, these studies were not performed in the context of NEDD8.

Accumulating evidence suggests that relative expression and stability of the different isoforms of p73 may contribute to a role in tumor suppression (48). TAp73 isoforms contain an N-terminal transactivation domain that is absent in the corresponding ∆N isoforms (due to the use of an alternative promoter within intron 3 or from alternative N-terminal splicing). Thus, although both the TA and ∆N isoforms can bind p53 DNA-binding sites, only TA isoforms can transactivate promoters of p53-target genes and induce apoptosis (1, 3, 8, 10). The ∆N isoforms act as dominant-negative inhibitors for TA isoforms of all three p53 family members by forming hetero-oligomers that generate an abortive transcriptional complex or by competing directly for p53-DNA-binding sites (5–10). As a result, the anti-apoptotic ∆N isoforms can block chemotherapy-induced apoptosis in tumor cells that retain wild type p53 (8, 10). In addition, elevated expression of ∆N isoforms have been associated with tumor progression, poor prognosis, and increased cancer recurrence (10, 12, 16, 17). The tumor suppressor role for TAp73 has been supported by the findings that TAp73 is induced by a wide variety of chemotherapeutic agents (49–51) and that blocking TAp73 function leads to chemoresistance (19, 52) and survival of squamous carcinoma cells (53). Thus, the role of p73 in tumorigenesis may lie in the relative expression of TA and ∆N isoforms. However, post-translational modifications differentially governing the expression and/or activity of TA and ∆Np73 isoforms have yet to be clearly characterized.

Here, we report the first evidence of a covalent post-translational regulation exclusively targeting the TA isoform of p73. We have demonstrated that TAp73β, but not ∆Np73β, is covalently modified by NEDD8 in an Mdm2-dependent manner. The failure of ∆Np73β neddylation is likely due to the absence of the critical Mdm2-binding domain located within the N terminus. In addition, a NEDD8-specific cysteine protease NEDP1 deconjugated neddylated TAp73β and an intact NEDD8 pathway attenuated TAp73β transcriptional activity in ts41 CHO cells. Furthermore, the expression of Mdm2 with NEDD8, but not with a conjugation-defective NEDD8ΔGG mutant, promoted accumulation of NEDD8-modified TAp73β in the cytoplasm. These results, taken together, suggest that neddylation of TAp73β via Mdm2 may exert its inhibitory effect on TAp73β transcriptional activity, at least in part, by altering its subcellular localization.

Mdm2 is one of the major regulators of p53 stability via the ubiquitin pathway. Interestingly, Mdm2, rather than promoting the degradation of TAp73, stabilizes TAp73 (33–35). The mechanism underlying Mdm2-mediated stabilization of
Mdm2 Mediates TAp73 Neddylation

TAp73 is currently unknown. We have noted increased levels of TAp73β upon ectopic expression of Mdm2 and/or NEDD8 (see Figs. 1 and 3; data not shown). It is, therefore, tempting to speculate that Mdm2-mediated neddylation of TAp73 results, not only in subcellular redistribution of TAp73 but also in its stabilization. Recently, Bernassola et al. (54) have shown that p73α accumulated under the non-permissive temperature in ts41 cells, suggesting a role of the NEDD8 pathway in the stabilization of p73α. In the context of our study, there are several reasons to consider that may explain why increased Mdm2-mediated stabilization of TAp73 is associated with decreased transactivation function of TAp73. The direct binding of Mdm2 to the N-terminal transactivation domain and/or competitive disruption of p300/CBP binding to the N-terminal transactivation domain by Mdm2 may inhibit TAp73 transcriptional activity (35). Furthermore, it is formally possible that Mdm2 regulates NEDD8 modification of lysyl residues (Lys321, Lys327, and Lys331), which are targets of acetylation by transcriptional co-activators p300/CBP (28). Thus, there are multiple modes of TAp73 regulation mediated by Mdm2. The elucidation of the precise molecular mechanisms that orchestrate the various post-translational regulatory modifications and the molecular signals that initiate Mdm2-mediated neddylation influencing the function of TAp73 are outstanding questions that remain to be resolved.

Acknowledgments—We thank the members of the Irwin and Ohh labs and comments. We also thank Angela Mabb in the laboratory of Dr. Shigeki Miyamoto at the University of Wisconsin-Madison for her technical assistance.

REFERENCES

1. Jost, C. A., Marin, M. C., and Kaelin, W. G., Jr. (1997) Nature 389, 191–194
2. Kaghad, M., Bonnet, H., Yang, A., Creancier, L., Biscan, J.-C., Valient, A., Minty, A., Chalon, P., Leisal, J.-M., Dumont, X., Ferrara, P., McKeon, F., and Caput, D. (1997) Cell 90, 809–819
3. Marin, M. C., Jost, C., Irwin, M. S., DeCaprio, J. A., Caput, D., and Kaelin, W. G. (1998) Mol. Cell. Biol. 18, 6316–6324
4. Irwin, M. S., and Miller, F. D. (2004) J. Cell. Physiol. 210, 816–817
5. Grazzini, S., Chalas, E., and Moll, U. M. (2002) Cancer Res. 62, 636–641
6. Nakagawa, T., Takahashi, M., Ozaki, T., Watanabe Ki, K., Todo, S., Mizuguchi, H., Hayakawa, T., and Nakagawa, A. (2002) Mol. Cell. Biol. 22, 2575–2585
7. Pozniak, C. D., Radinovic, S., Yang, A., McKeon, F., Kaplan, D. R., and Miller, F. D. (2000) Science 289, 304–306
8. Stiewe, T., Theseling, C. C., and Putzer, B. M. (2002) J. Biol. Chem. 277, 14177–14185
9. Yang, A., Walker, N., Bronson, R., Kaghad, M., Oosterwegel, M., Bonnin, J., Vagner, C., Bonnet, H., Dikkes, P., Sharpe, A., McKeon, F., and Caput, D. (2000) Nature 404, 99–103
10. Akaka, C. A., Slade, N., Erster, S. H., Sansome, C., Joseph, T. W., Pearl, M., Chalas, E., and Poll, U. M. (2002) J. Exp. Med. 196, 765–780
11. Concin, N., Becker, K., Slade, N., Erster, S., Mueller-Holzner, E., Ulmer, H., Daxenbichler, G., Zeimet, A., Zeillinger, R., Martin, S. J., Latchman, D. S., Knight, R. A., Melino, G., and De Lauretiis, V. (2000) Mol. Cell. Biol. 20, 8458–8467
12. Honda, R., Tanaka, H., and Yasuda, H. (1997) FEBS Lett. 420, 25–27
13. Ruben, M., Schilling, T., Sayan, A. E., Kairat, A., Lorenz, K., Schulze-Bergkamen, H., Oren, M., Koch, A., Tannapfel, A., Stremlow, W., Melino, G., and Krammer, P. H. (2005) Cell Death Differ. 12, 1564–1577
14. Ramirez, S., Pries, C., and Cordon-Cardo, C. (2003) Mol. Cell. Biol. 23, 8161–8171
15. Pozniak, C. D., Radinovic, S., Yang, A., McKeon, F., Kaplan, D. R., and Miller, F. D. (2000) Science 289, 304–306
16. Lohrum, M. A., Woods, D. B., Ludwig, R. L., Balint, E., and Vousden, K. H. (2005) Mol. Cell. Biol. 25, 829–832
17. Balint, E., Bates, S., and Vousden, K. H. (1999) Oncogene 18, 3923–3929
18. Santos, A., Merlo, P., Pediconi, N., Fulco, M., Sartorelli, V., Cole, P. A., Fontemaggi, G., Fanciulli, M., Schultz, L., Blandino, G., Balsano, C., and Levervo, M. (2002) Mol. Cell 9, 175–186
19. Gonzalez, S., Pries, C., and Cordon-Cardo, C. (2003) Mol. Cell. Biol. 23, 8161–8171
20. Balint, E., Bates, S., and Vousden, K. H. (1999) Oncogene 18, 3923–3929
21. Vagner, C., Bonnet, H., Dikkes, P., Sharpe, A., McKeon, F., and Caput, D. (2000) Nature 404, 99–103
22. Yang, A., Walker, N., Bronson, R., Kaghad, M., Oosterwegel, M., Bonnin, J., Vagner, C., Bonnet, H., Dikkes, P., Sharpe, A., McKeon, F., and Caput, D. (2000) Nature 404, 99–103
23. Nakagawa, T., Takahashi, M., Ozaki, T., Watanabe Ki, K., Todo, S., Mizuguchi, H., Hayakawa, T., and Nakagawa, A. (2002) Mol. Cell. Biol. 22, 2575–2585
24. Stiewe, T., Theseling, C. C., and Putzer, B. M. (2002) J. Biol. Chem. 277, 14177–14185
25. Yang, A., Walker, N., Bronson, R., Kaghad, M., Oosterwegel, M., Bonnin, J., Vagner, C., Bonnet, H., Dikkes, P., Sharpe, A., McKeon, F., and Caput, D. (2000) Nature 404, 99–103
26. Akaka, C. A., Slade, N., Erster, S. H., Sansome, C., Joseph, T. W., Pearl, M., Chalas, E., and Poll, U. M. (2002) J. Exp. Med. 196, 765–780
27. Concin, N., Becker, K., Slade, N., Erster, S., Mueller-Holzner, E., Ulmer, H., Daxenbichler, G., Zeimet, A., Zeillinger, R., Martin, S. J., Latchman, D. S., Knight, R. A., Melino, G., and De Lauretiis, V. (2000) Mol. Cell. Biol. 20, 8458–8467
James, N., McGregor, J. M., Harwood, C. A., Yulug, I. G., Voussden, K. H., Allday, M. J., Gusterson, B., Ikawa, S., Hinds, P. W., Crook, T., and Kaelin, W. G., Jr. (2000) Nat. Genet. 25, 47–54

Bottger, A., Bottger, V., Garcia-Echeverria, C., Chene, P., Hochkeppel, H. K., Sampson, W., Ang, K., Howard, S. F., Picksley, S. M., and Lane, D. P. (1997) J. Mol. Biol. 269, 744–756

Kussie, P. H., Gorina, S., Marechal, V., Elenbaas, B., Moreau, J., Levine, A. J., and Pavletich, N. P. (1996) Science 274, 948–953

Oberst, A., Rossi, M., Salomoni, P., Pandolfi, P. P., Oren, M., Melino, G., and Bernassola, F. (2005) Biochem. Biophys. Res. Commun. 331, 707–712

Agami, R., Blandino, G., Oren, M., and Shaul, Y. (1999) Nature 399, 809–813

Gong, J., Costanzo, A., Yang, H., Melino, G., Kaelin, W. G., Levero, M., and Wang, J. (1999) Nature 399, 806–808

Yuan, Z.-M., Shioya, H., Ishiko, T., Sun, X., Gu, J., Huang, Y., Lu, H., Kharbanda, S., Weichselbaum, R., and Kufe, D. (1999) Nature 399, 814–817

Bergamaschi, D., Gasco, M., Hiller, L., Sullivan, A., Syed, N., Trigiante, G., Yulug, I., Merlano, M., Numico, G., Comino, A., Attard, M., Reelfs, O., Gusterson, B., Bell, A. K., Heath, V., Tavassoli, M., Farrell, P. J., Smith, P., Lu, X., and Crook, T. (2003) Cancer Cell 3, 387–402

Rocco, J. W., Leong, C. O., Kuperwasser, N., DeYoung, M. P., and Ellisen, L. W. (2006) Cancer Cell 9, 45–56

Bernassola, F., Salomoni, P., Oberst, A., Di Como, C. J., Pagano, M., Melino, G., and Pandolfi, P. P. (2004) J. Exp. Med. 199, 1545–1557