Regulation of the MDM2-p53 Pathway by Ribosomal Protein L11 Involves a Post-ubiquitination Mechanism

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Abstract

Inhibition of the MDM2-p53 feedback loop is critical for p53 activation in response to cellular stresses. The ribosomal proteins L5, L11, and L23 can block this loop by inhibiting MDM2-mediated p53 ubiquitination and degradation in response to ribosomal stress. Here, we show that L11, but not L5 and L23, leads to a drastic accumulation of ubiquitinated and native MDM2. This effect is dependent on the ubiquitin ligase activity of MDM2, but not p53, and requires the central MDM2 binding domain (residues 51–108) of L11. We further show that L11 inhibited 26 S proteasome-mediated degradation of ubiquitinated MDM2 in vitro and consistently prolonged the half-life of MDM2 in cells. These results suggest that L11, unlike L5 and L23, differentially regulates the levels of ubiquitinated p53 and MDM2 and inhibits the turnover and activity of MDM2 through a post-ubiquitination mechanism.

The tumor suppressor protein p53 is a transcription factor activated in response to stress to induce expression of its target genes. The proteins encoded by these genes then mediate multiple cellular responses, such as cell cycle arrest, apoptosis, differentiation, cell senescence, or DNA repair (1). Also, p53 can directly trigger mitochondria-mediated apoptosis in response to DNA damage (2–4). The tumor suppression function of p53 is well reflected in the fact that more than half of human tumors harbor mutations in the p53 gene, and many others retain impaired function of the p53 pathway (5,6). Because of its inhibitory effect on cell growth, p53 is maintained at a low steady-state level and in an inert form in physiological conditions. This duty is mainly fulfilled by the E3 ubiquitin ligase MDM2 that mediates p53 constant degradation through a ubiquitin-dependent proteasome pathway (7,8). The mdm2 gene itself is a downstream target of p53, thus forming a tight autoregulatory feedback loop (9–11). Consistent with this notion, gene amplification and overexpression of MDM2 have also been shown in a variety of tumors, particularly in soft tissue sarcomas, lymphomas, and breast and lung cancers (12–16). Interfering with the MDM2-p53 feedback loop leads to p53 activation, ultimately preventing neoplasia. One example of this regulation is alternative reading frame (ARF) (p14ARF in human, p19ARF in mouse)-mediated inhibition of this loop in response to

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2The abbreviations used are: E3, ubiquitin-protein isopeptide ligase; ARF, alternative reading frame; E1, ubiquitin activating enzyme (UBA); E2, ubiquitin carrier protein; HA, hemagglutinin; Ni-NTA, nickel nitritotriacetic acid; GFP, green fluorescent protein; MEF, murine embryonic fibroblast cell.
overexpression of oncogenes such as c-Myc and RAS (17,18). Also, in response to DNA-damaging agents, N-terminal serine/threonine phosphorylation at the MDM2 binding domain of p53 prevents MDM2-p53 interaction and activates p53 (19–23). Hence, the MDM2-p53 loop presents as a central regulatory point for the cellular response to a multitude of environmental as well as internal stressors.

Increasing evidence shows that the MDM2-p53 feedback loop can also be regulated by ribosomal stress. Fine coordination between ribosomal biogenesis and other cellular functions such as the cell cycle and differentiation is important for normal cell growth (24,25). Impeding ribosomal biogenesis would generate ribosomal stress that activates p53 to stop cell growth. Examples of such stress include inhibition of ribosomal RNA (rRNA) synthesis and processing by a low dose (< 5 nM) of actinomycin D or overexpression of a dominant-negative mutant of the rRNA processing factor Bop1 (26,27). Malfunctions of ribosomal biogenesis have also been proposed to correlate to human cancers (28). Recently, we and others reported that ribosomal proteins L5, L11, and L23 interacts with MDM2 and inhibited the MDM2-p53 feedback loop in response to ribosomal stress, such as treatment with low dose actinomycin D, serum starvation, or possibly small interference RNA-induced reduction of L23 (29–34). Interestingly, these L proteins as well as the tumor suppressor protein ARF are primarily nucleolar proteins. Disruption of the nucleolus appears to be a common event in stress-induced p53 activation pathways (35). Thus, releasing small protein molecules such as the ribosomal L proteins from the nucleolus leads to p53 activation in response to ribosomal stress.

Although ectopic expression of L5, L11, or L23 can inhibit MDM2-mediated p53 ubiquitination and degradation (29–33), the detailed mechanisms underlying this effect remain undetermined. Also, little is known about the effect of these L proteins on MDM2 stability and ubiquitination. We have begun to address these issues by performing a series of cellular and biochemical analyses. In this study, we found that unlike L5 and L23, which drastically inhibited ubiquitination of both p53 and MDM2, L11 slightly inhibited MDM2-mediated p53 ubiquitination but markedly increased the ubiquitinated species and the steady-state level of MDM2 in cells. This effect was dependent on the ubiquitin ligase activity of MDM2, but not p53, and required the central MDM2-binding domain of L11. Interestingly, L11 inhibited proteasome-mediated degradation of ubiquitinated MDM2 in vitro. These results suggest that L11 differentially regulates the levels of ubiquitinated p53 and MDM2 through a post-ubiquitination and proteasome-dependent mechanism.

**MATERIALS AND METHODS**

**Cell Lines, Plasmids, and Antibodies**

Human lung small cell adenocarcinoma H1299 cells, human osteosarcoma U2OS cells, and mouse p53−/−/mdm2−/− MEFs were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) fetal bovine serum, 50 units/ml penicillin, and 0.1 mg/ml streptomycin at 37 °C in a 5% CO2 humidified atmosphere as previously described (33).

The FLAG-tagged L5, L11, and L23 expression plasmids have been described previously (30,32,33). All the deletion mutants of L11 were generated using PCR and cloned into pcDNA3-2-FLAG vector. The HA-MDM2 expression vector has been described (33). The full-length p14ARF (ARF, hereafter) was amplified using PCR and inserted into the pcDNA3-V5 vector at BamHI and EcoRI sites to generate pcDNA3-V5-ARF. The primers were 5′-CGGGAGATTCTAGTGGTGCAGGTTCCTTGGTG-3′ and 5′-CCGGGATTCTACGCGAGGCGACAC-3′. The MDM2 mutant with a point mutation at position 464 from cysteine to alanine (MDM2C464A) was generously provided by Dr. Karen H. Vousden (36).
Anti-FLAG (Sigma), anti-p21 (NeoMarkers), anti-MDM2 (N20), and anti-p53 (DO-1, Santa Cruz) were purchased. Anti-MDM2 (2A10) and anti-HA (12CA5) have been described (33).

Purified Proteins for in Vitro Ubiquitination Assay
Recombinant FLAG-MDM2 full-length protein was purified from baculovirus-infected SF9 insect cells as described (37). His-tagged L11, L5, and L23 were expressed in *Escherichia coli* and purified through a nickel nitrilotriacetic acid (Ni-NTA, Qiagen) column as previously described (32). Purified rabbit ubiquitin activating enzyme (UBA) E1, purified human recombinant E2 (UbcH5a), and recombinant human ubiquitin were purchased from Boston Biochem.

Cotransfection, Immunoblot, and Co-immunoprecipitation Analyses
H1299, U2OS, or *p53*−/−/mdm2−/− MEFs cells were transfected with plasmids as indicated in each figure legend using Lipofectin following the manufacturer’s protocol (Invitrogen). Cells were harvested at 48 h post-transfection and lysed in lysis buffer consisting of 50 mM Tris/HCl (pH 8.0), 0.5% Nonidet P-40, 1 mM EDTA, 150 mM NaCl, and 1 mM phenylmethylsulfonyl fluoride. Equal amounts of clear cell lysate were used for immunoblot analysis as described previously (33). Immunoprecipitation was conducted using antibodies as indicated in the figure legends and described previously (33). Beads were washed with lysis buffer twice, once with SNNTE buffer (50 mM Tris/HCl (pH 7.4), 5 mM EDTA, 1% Nonidet P-40, 500 mM NaCl, and 5% sucrose), and once with radioimmune precipitation assay buffer (50 mM Tris/HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, 0.1% SDS, and 1% (w/v) sodium deoxycholate). Bound proteins were detected by immunoblot using antibodies as indicated in the figure legends.

In Vivo Ubiquitination Assay
In *vivo* ubiquitination assays were conducted as previously described (33). Briefly, H1299 cells or *p53*−/−/mdm2−/− MEFs (60% confluence/100-mm plate) were transfected with combinations of the following plasmids as indicated in the figure legends: His6-ubiquitin (2 μg), p53 (1 μg), HA-MDM2 (2 μg), FLAG-L5 (2 μg), FLAG-L11 (2 μg), FLAG-L23 (2 μg), V5-ARF (2 μg) using Lipofectin (for H1299 cells), or Lipofectamine 2000 (for *p53*−/−/mdm2−/− MEFs) (Invitrogen). For inhibition of proteasome-mediated protein degradation, the cells were treated with 20 μM MG132 for 8 h before harvest. Forty-eight hours after transfection cells from each plate were harvested and split into two aliquots, one for immunoblot and the other for ubiquitination assays. Cell pellets were lysed in buffer I (6 M guanidinium-HCl, 0.1 mol/liter Na2HPO4/NaH2PO4, 10 mmol/liter Tris-HCl (pH 8.0), 10 mmol/liter β-mercaptoethanol) and incubated with Ni-NTA beads at room temperature for 4 h. Beads were washed once each with buffer I, buffer II (8 mol/liter urea, 0.1 mol/liter Na2HPO4/NaH2PO4, 10 mmol/liter Tris-HCl (pH 8.0), 10 mmol/liter β-mercaptoethanol), and buffer III (8 mol/liter urea, 0.1 mol/liter Na2HPO4/NaH2PO4, 10 mmol/liter Tris-HCl (pH 6.3), 10 mmol/liter β-mercaptoethanol). Proteins were eluted from the beads in buffer IV (200 mmol/liter imidazole, 0.15 mol/liter Tris-HCl (pH 6.7), 30% (v/v) glycerol, 0.72 mol/liter β-mercaptoethanol, and 5% (w/v) SDS). Eluted proteins were analyzed by immunoblot with monoclonal anti-p53 (DO-1), anti-HA, or anti-MDM2 (2A10) antibodies.

In Vitro Ubiquitination Reactions
*MDM2 in vitro* auto-ubiquitination reactions were performed in ubiquitination buffer (25 mM Hepes (pH 7.4), 10 mM NaCl, 3 mM MgCl2, 0.05% TritonX-100, freshly added 0.5 mM dithiothreitol) with 100 ng of UBA-E1, 25 ng of UbcH5a, and 5 ng of ubiquitin and were incubated for 120 min at 37 °C. A total of 10 ng of purified FLAG-MDM2 was used in each
reaction. The mixture was either resolved by a SDS-PAGE gel followed by immunoblot using anti-MDM2 antibody or subjected to proteasome-dependent degradation assays.

**In Vitro 26 S Proteasome-dependent Degradation Assay**

A total of 1 μg of purified 26 S proteasome (human erythrocytes, BIOMOL) preincubated with proteasome inhibitors (10 μM MG132, 10 μM lactacystin, 10 μM clasto-lactacystin-β-lactone) for 30 min at 4 °C where indicated was added to ubiquitination reactions along with 3 mM Mg-ATP (Boston Biochem) and further incubated at 37 °C for 5 h. To test the effect of L11, L5, and L23 on proteasome-dependent degradation of MDM2, ubiquitinated MDM2 was preincubated with purified L11, L5, and L23 protein for 30 min at 4 °C in ubiquitination buffer before the addition to the degradation reaction.

**RESULTS**

**Differential Effect of L11, L5, L23, and ARF on the Levels of Ubiquitinated MDM2 and p53**

In an attempt to elucidate the mechanisms by which ribosomal proteins regulate the stability of MDM2 and p53, we determined the effect of ribosomal proteins L5, L11, and L23 on the ubiquitination of MDM2 and p53 in p53-deficient H1299 cells using ARF as a positive control for a factor known to stabilize p53 when exogenously introduced into cells. The cells were transfected with plasmids encoding MDM2 and p53 alone or with either one of the L proteins or ARF. As a control, the cells transfected with MDM2 and p53 only were treated with 20 μM concentrations of the proteasome inhibitor MG132 for 8 h before harvesting. The cells were harvested 48 h after transfection, and *in vivo* ubiquitination assays were conducted as described under “Materials and Methods.” As shown in Fig. 1A and as expected (36,38), cotransfection of MDM2 with p53 resulted in p53 ubiquitination and drastic reduction of its protein level (compare lane 3 to lane 2). Consistent with our previous results (32,33), ectopic expression of L5 and L23 markedly inhibited the ubiquitination of both p53 and MDM2 and consequently protected p53 degradation by MDM2 (lanes 5 and 7 of Figs. 1, A and B). However, overexpression of L11 dramatically increased the ubiquitinated species of p53 and MDM2 as well as their protein levels (lane 6 of Figs. 1, A and B). This effect was similar to that of ARF (lane 4 of Figs. 1, A and B) as reported previously (39). The enhancement of the level of ubiquitinated MDM2 and p53 species as well as their protein levels by L11 and ARF was not due to the variation in transfection efficiency because the GFP protein level in each transfectant was equivalent (Fig. 1C). Of note, the effect of L11 and ARF on stabilizing p53 and MDM2 was much more prominent than that of L5 and L23 (Fig. 1, A and B). Interestingly, the effect of L11 and ARF on the ubiquitination of MDM2 and p53 was similar to that of MG132 treatment (lane 8, Fig. 1, A and B), although to different extents. L11 was more effective than MG132 in enhancing the level of ubiquitinated MDM2 species. This might be due to the specific inhibition of 26 S proteasome-mediated degradation of ubiquitinated MDM2 by L11 (see Fig. 3C). These results suggest that these ribosomal proteins may utilize different mechanisms to regulate the MDM2 and p53 ubiquitination and proteasomal pathways although all these nucleolar proteins can stabilize p53 upon overexpression (Fig. 1).

However, we were unsure whether the changes in ubiquitinated p53 or MDM2 species induced by the ribosomal L proteins were due to the direct effect of these proteins on the ubiquitin ligase activity of MDM2. To determine the effect of the L proteins and ARF on the MDM2 ubiquitin ligase activity with p53 and MDM2 as substrates, we performed a set of transfections similar to that in Fig. 1, but 20 μM MG132 was used to block proteasomal degradation so that we could compare ubiquitination at similar protein levels. As shown in Fig. 2, L5 and L23 again dramatically inhibited ubiquitination of both p53 and MDM2 (lanes 5 and 7, Fig. 2, A and B), indicating that these two L proteins may directly inhibit MDM2 ubiquitin ligase activity as previously described (32,33). By striking contrast, L11, like ARF (39), which slightly
reduced MDM2-mediated ubiquitinated species of p53 (lanes 4 and 6, Fig. 2A), still markedly increased the ubiquitinated species of MDM2 (lanes 4 and 6, Fig. 2B). This difference was not due to different transfection efficiencies among the different conditions, as all of the exogenous proteins were expressed equally well (Fig. 2C). These results suggest that L11 differentially regulates the levels of ubiquitinated species of p53 and MDM2, causing decreased p53 ubiquitinated species but increased MDM2 ubiquitinated species.

L11 Inhibits in Vitro Degradation of Ubiquitinated MDM2 by 26 S Proteasome

Because overexpression of L11, but not L5 and L23, led to similar enhancing effects on the levels of ubiquitinated MDM2 as that of MG132 (Fig. 1) or overexpression of ARF (39), we next wanted to determine whether L11, like MG132, could block proteasome-mediated degradation of ubiquitinated MDM2. To test this possibility, we performed an in vitro MDM2 autoubiquitination reaction as shown in Fig. 3A. In the presence of E1, E2, and ubiquitin, MDM2 efficiently ubiquitinated itself (lane 3) as expected (40, 41). To test if ubiquitination of MDM2 is required for its degradation by 26 S proteasome, we incubated purified MDM2 with 26 S proteasome or buffer and then assayed for the level of MDM2. As shown in Fig. 3B, purified MDM2 is not degraded by 26 S proteasome, indicating that ubiquitination is essential for proteosomal-mediated degradation of MDM2. Next, the ubiquitinated MDM2 was then used for 26 S proteasome-mediated degradation assays. As shown in Fig. 3C, the addition of purified 26 S proteasome caused a marked decrease in the amounts of MDM2-ubiquitin conjugates as well as a decrease of native (non-modified) MDM2 (compare lane 2 to lane 1). These effects were efficiently blocked by the addition of MG132 (lane 3), suggesting that the ubiquitinated MDM2 can be efficiently degraded through the proteasome system in our assays. Consistent with the above results, L11 (lanes 4–5), but not L5 (lanes 6–7) and L23 (lanes 8–9), drastically inhibited the degradation of ubiquitinated MDM2 by the 26 S proteasome. These results suggest that L11 directly blocked 26 S proteasome-mediated degradation of MDM2-ubiquitin conjugates. These results explain the accumulation of ubiquitinated species of MDM2 by L11 in cells (Fig. 2).

L11 Also Stabilizes p53 in Cells

It has been shown that ectopic expression of L11 leads to elevated p53 protein levels and transcriptional activity in cells (29, 30). Consistently, we also observed that both endogenous p53 and MDM2 proteins were dramatically increased upon L11 overexpression in p53 proficient U2OS cells (Fig. 4A). However, the effect of L11 on p53 stability has not been determined. Therefore, we transfected U2OS cells with or without FLAG-L11 plasmids. Forty-eight hours after transfection, the cells were treated with cyclohexamide to stop protein synthesis in cells. The cells were then harvested at different time points and subjected to immunoblot to determine endogenous p53 levels (Fig. 4B). The protein levels were determined by measuring the intensity of each band and normalized with expression of GFP. As shown in Figs. 4, B and C, the half-life of p53 prolonged from ~25 min in empty vector transfected cells to more than 2 h in the cells overexpressing FLAG-L11. These results are consistent with the notion that L11 inhibited MDM2-mediated p53 ubiquitination (Fig. 2) and demonstrate that the increased level of p53 in the presence of ectopically expressed L11 is due to p53 stabilization by this ribosomal protein. We also observed that L11 significantly increased the half-life of MDM2 in the same assay (data not shown). Therefore, L11 can also stabilize MDM2 as well.

Increment of Ubiquitinated Species and Stability of MDM2 by L11 is p53-independent

L11 can directly bind to MDM2 (32) but not p53 (data not shown). Therefore, it is likely that stabilization of p53 by L11 is through the inhibitory effect of L11 on MDM2. However, is the L11-induced increase in ubiquitinated species and stability of MDM2 dependent on p53? To
address this issue, we examined the effect of L11 on the half-life of MDM2 and its autoubiquitination in p53-deficient H1299 cells. Indeed, as shown in Fig. 5, A and B, the half-life of ectopic MDM2 protein increased from ~20 min in the cells transfected with a control vector to ~2 h in the FLAG-L11-expressing cells. Because L11 is a component of the large subunit ribosomal complex, we also tested whether overexpression of L11 could enhance the translation of MDM2. H1299 cells were transfected with HA-MDM2 with or without FLAG-L11. Forty-eight hours post-transfection, the cells were starved in the medium without methionine and then pulse-labeled with $^{[35}\text{S}]$methionine for 15 min. Equal amounts of total protein were immunoprecipitated with the anti-HA antibody. Total MDM2 protein and $^{35}\text{S}$-labeled MDM2 levels were determined by immunoblot and autoradiography, respectively. As shown in Fig. 5C, the de novo MDM2 protein translation was not affected by FLAG-L11 overexpression (top and bottom panels), whereas the increment of the MDM2 protein level was clearly evident (middle panel). Consistently, L11 also elevated the ubiquitinated species of MDM2 in p53-deficient H1299 cells (Fig. 5D) and in p53/mdm2 double knock-out MEF cells (Fig. 6). Taken together, these results demonstrate that L11 can stabilize MDM2 and enhance its ubiquitinated species in a p53-independent manner.

Because the above in vitro and in vivo ubiquitination and degradation assays indicated that the prime effect of L11 on MDM2 stability might be through inhibition of proteasome-mediated degradation of ubiquitinated MDM2 (Figs. 1–3), another question is whether L11, like MG132, plays a global role in preventing proteasome-mediated protein turnover. To test this idea, we examined the specificity of L11-dependent effect on protein turnover in cells. As shown in Fig. 5E, L11 did not increase the level of p63 (lanes 1 and 2) in H1299 cells. Interestingly, L11 did not increase the level of exogenous p53 in p53/mdm2 double knock-out MEF cells either (lanes 3 and 4), indicating that the effect of L11 on p53 stability is MDM2-dependent. These results suggest that L11 specifically stabilizes MDM2 through inhibition of its proteasome-mediated degradation, although it is possible that L11 may have a selective effect on other protein targets that were not tested here.

### Enhancement of Ubiquitinated MDM2 Species by L11 Is Dependent on MDM2 Ubiquitin Ligase Activity

To determine whether the L11-induced increase of ubiquitinated species of MDM2 is dependent on the MDM2 E3 ubiquitin ligase activity, we performed in vivo ubiquitination assays using an MDM2 mutant with substitution of cysteine 464 by alanine (MDM2\textsuperscript{C464A}). This cysteine residue is essential for the ubiquitination of p53 by MDM2, and the C464A mutation abolishes the ubiquitin E3 ligase activity of MDM2 (36,38,42), suggesting that MDM2 ubiquitination in cells is executed through its own E3 ubiquitin ligase activity. First, we transfected H1299 cells with His-ubiquitin together with either a wild-type or a C464A mutant MDM2 plasmid in the presence or absence of FLAG-L11. Ubiquitinated MDM2 was pulled down with Ni-NTA beads followed by immunoblot with the anti-MDM2 antibody. As shown in Fig. 6A, the cells transfected with MDM2 in the absence of FLAG-L11 showed a detectable level of ubiquitinated MDM2 (lane 2 of the top panel). By contrast, the MDM2\textsuperscript{C464A} mutant showed only trace amounts of ubiquitination (lane 4), suggesting that MDM2 ubiquitination in cells is executed through its own E3 ubiquitin ligase activity. Upon overexpression of L11, ubiquitinated species of wild-type MDM2 were remarkably accumulated (lane 3 of the top panel). Surprisingly, ubiquitinated species of MDM2\textsuperscript{C464A} mutant were also dramatically enhanced by FLAG-L11 (lane 5 compared with lane 4 of the top panel). Two possibilities may account for this effect; L11 may enhance MDM2\textsuperscript{C464A} ubiquitination by other ubiquitin ligases or the enhanced MDM2\textsuperscript{C464A} ubiquitination may be mediated by endogenous MDM2, which is expressed at a relative low level in p53-free H1299 cells. To test these possibilities, we performed similar transfections and in vivo ubiquitination assays as mentioned in Fig. 6A in p53\textsuperscript{+/−}/mdm2\textsuperscript{+/−} MEF cells. As shown in Fig. 6B, the cells

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transfected with MDM2 in the absence of FLAG-L11 showed a detectable level of ubiquitinated MDM2 (lane 2 of the top panel). By contrast, the MDM2C464A mutant showed no detectable ubiquitination (lane 4), further suggesting that MDM2 ubiquitination is dependent on its own functional ubiquitin ligase activity. Upon overexpression of L11, ubiquitinated species of wild-type MDM2 were remarkably accumulated (lane 3 of the top panel). However, ubiquitinated MDM2C464A was hardly detectable in this mdm2/p53 null MEF cell line regardless of the presence of FLAG-L11 or not (lanes 4 and 5 of the top panel in Fig. 6B). These results indicate that stabilization and the increased level of ubiquitinated species of MDM2 by L11 are dependent on the intrinsic ubiquitin E3 ligase activity of MDM2.

The Central Domain of L11 Binds to MDM2 and Is Required for MDM2 Stabilization, Enhanced Level of the Autoubiquitinated Species of MDM2, and Activation of p53

To determine which domain of L11 is important for the above regulation of MDM2 by L11, we generated a set of deletion mutants of L11 that were FLAG-tagged as illustrated in Fig. 7B. Because the central region (amino acids 63–125) of L11 has been shown to bind to MDM2 (29), we wanted to verify this result while also hoping to further narrow down the MDM2 binding domain. To this end we performed a series of co-immunoprecipitation assays using the newly generated L11 deletion mutants. As shown in Fig. 7A, HA-MDM2 was co-immunoprecipitated with C-terminal-deleted mutants L111–108 (lane 5), L111–125, and L111–143 (data not shown) using anti-FLAG antibodies although the binding efficiency of these mutants decreased in comparison with that of wild-type L11 (lane 3). However, L111–65 did not bind to MDM2 at all (lane 4). An N-terminal deletion mutant (L1151–178, lane 6), but not other N-terminal deletion mutants L11109–178 (lane 7) and L1166–178 (data not shown), was also co-immunoprecipitated with MDM2. These results, as summarized in Fig. 7B, indicate that the central domain of L11 consisting of amino acids 51–108 is essential for MDM2-binding. Consistently, as shown in the second bottom panel in Fig. 7A, both the MDM2 binding N-terminal (L1151–178)-and C-terminal (L111–108)-deleted L11 mutants, similar to wild-type L11 (lane 3), stabilized exogenous MDM2 (lanes 5 and 6) although to a lesser extent, whereas other mutants, which did not interact with MDM2 (Figs. 7, A and B), failed to stabilize MDM2 (lanes 4 and 7 and data not shown). Thus, the central domain of L11 consisting of residues 51–108 is required for L11 to stabilize MDM2 in cells. Furthermore, we examined the effect of some of these L11 deletion mutants on MDM2 autoubiquitination. Consistently, only the MDM2 binding deletion mutant of L11 (L111–108), but not the MDM2 binding-deficient mutants (L111–65 or L11109–178), enhanced the level of ubiquitinated species of MDM2 in cells (Fig. 7C). These results suggest that the central domain of L11 (amino acids 51–108) is required for L11 to interact with and to stabilize MDM2 as well as to enhance the ubiquitinated species of MDM2 in cells.

To further determine whether the central domain of L11 is required for activation of p53, we transfected U2OS cells with wild-type or different deletion mutants of L11 and then examined the endogenous levels of p53, p21, and MDM2. Indeed, L111–108, but not L111–65, L11109–178, and L11126–178, induced higher p53 levels (top panel) and stimulated its transcriptional activity as measured by the induction of the p53 target genes, p21 and MDM2 (Fig. 7D). Of note, the lesser effect of the L111–108 mutant on p53 activation (Fig. 7D) was correlated with its reduced MDM2 binding activity (Fig. 7A). In summary, these results indicate that the central MDM2 binding domain of L11 is required for p53 activation.

DISCUSSION

Inhibition of the MDM2-p53 feedback loop is important for p53 induction and activation in response to cellular stress signals. This inhibition can take place by either interfering with the physical interaction between MDM2 and p53 or directly inhibiting the MDM2 E3 ligase
activity (43,44). Significantly, these cellular mechanisms have become targeting sites for identifying small molecule inhibitors of MDM2 as anti-tumor drug candidates (45–47). In cells, one of the well studied natural inhibitors of the MDM2-p53 feedback loop is the tumor suppressor ARF in response to high levels of oncoproteins, whereas ARF is believed to regulate rRNA processing in the nucleolus under normal situations (40,48–50). Likewise, several ribosomal proteins including L5, L11, and L23, which are usually assembled into the 80 S translation complex in the nucleolus and exported to the rough endoplasmic reticulum for protein synthesis, have also been recently identified as the cellular blockers of this MDM2-p53 loop in response to ribosomal stress (29–34). Therefore, further understanding the mechanisms by which these ribosomal proteins inhibit MDM2 function would provide useful information for recapturing p53 activation in MDM2 highly expressed cancer cells and, hence, for pharmacological study.

As a result of this effort, we have described here that these three ribosomal proteins appear to utilize different mechanisms to regulate MDM2 function despite forming a quadruple complex with MDM2 (32). Overexpression of L5, L11, or L23 has been shown to inhibit MDM2-mediated p53 ubiquitination (29,31–33). Here, we found that L23 (33) and L5 (Figs. 1B and 2B) inhibited MDM2 autoubiquitination in cells, suggesting that both of the proteins may stall MDM2-mediated p53 ubiquitination by inhibiting the ubiquitin ligase activity of MDM2. Although the detailed biochemical mechanism underlying this inhibition remains to be investigated, it is possible that L5 or L23 could recruit certain ubiquitin hydrolases, such as the recently reported herpesvirus-associated ubiquitin-specific protease (HAUSP) (51,52), leading to deubiquitination of both MDM2 and p53. Alternatively, L5 and L23 may directly inhibit the E3 ubiquitin ligase activity of MDM2 by concealing the accessibility of MDM2 or p53 to E2.

Unlike L23 and L5, L11 inversely enhanced ubiquitinated species of MDM2 (Figs. 1B) even in the presence of the proteasome inhibitor, MG132, in cells (Fig. 2B). Correspondingly, L11 induced higher steady-state levels of MDM2 and markedly elongated the half-life of MDM2 independently of p53 (Fig. 5). This induction was not due to the effect of highly and transiently expressed L11 on de novo protein synthesis (Fig. 5C). Instead, L11, but not L5 and L23, blocked the degradation of ubiquitinated MDM2 by 26 S proteasome in vitro (Fig. 3). These results strongly suggest that L11 inhibits proteasome-mediated degradation of ubiquitinated MDM2 through a post-ubiquitination mechanism. These effects of L11 are reminiscent of the effect of ARF (39) and similar to the effect of MG132 treatment (Fig. 1). However, the post-ubiquitination mechanism by which L11 increases MDM2 ubiquitin conjugates could be different from that of MG132. MG132 inhibits overall proteosomal activity, whereas L11 specifically inhibits proteasome-mediated degradation of ubiquitinated MDM2. Supporting this notion is that L11 did not stabilize other short-lived proteins such as p63 or exogenous p53 in p53/mdm2 double-knock MEF cells (Fig. 5E). Therefore, it is highly likely that L11 may not directly inhibit overall proteasome activity; instead, it may prevent the recruitment of ubiquitinated MDM2 to the proteasome, either through potential adaptor proteins or by concealing MDM2 binding site in the proteasome.

MDM2 is required for both ubiquitination and degradation of p53. Although the mechanism is not fully characterized, a post-ubiquitination role has been proposed for MDM2 in the p53 degradation pathway (53–55). For example, hHR23, a member of a proteasome-interacting protein family that contains ubiquitin-like and ubiquitin-associated domains (UbL-UBA proteins) was shown to associate with MDM2 and to regulate the targeting of MDM2 and p53 to the proteasome for degradation (53,54). Also, it has been recently shown that MDM2 directly binds to the C8 subunit of the 20 S proteasome and promotes Rb protein degradation in a proteasome-dependent manner (56). Thus, MDM2 may serve as a post-ubiquitination

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proteasome adaptor for its ubiquitinated substrates such as p53 with assistance of other proteasome-interacting proteins like hHR23.

MDM2 also mediates its own ubiquitination and proteasome-mediated degradation. Thus, it is possible that MDM2 also serves as a proteasome adaptor for its own degradation. In this scenario, MDM2 may be recruited to proteasome directly or through other cofactors, and then it may dimerize or oligomerize with ubiquitinated MDM2, leading to proteasome recognition and degradation of ubiquitinated MDM2. Our data as shown here suggest that L11-MDM2 interaction may inhibit this post-ubiquitination pathway, thus blocking proteasome-mediated MDM2 degradation. Although L11 also blocks MDM2-mediated p53 degradation in our experimental settings (Fig. 1), we did not observe further accumulation of ubiquitinated p53 when proteasome activity was blocked by MG132 in the setting of L11 expression (Fig. 2). This may be due to the inhibition of MDM2 ubiquitin ligase activity toward p53 (7,8).

Therefore, we propose that L11 may share a common post-ubiquitination mechanism to regulate MDM2-p53 pathway with ARF (39), hHR23 (53,54), and glycogen synthase kinase 3 (55) to stabilize and activate p53. Because L11-MDM2 binding is required for the L11 effect on enhancing autoubiquitinated species (Fig. 7), it is conceivable that the central L11 binding domain (amino acids 284–374) (29), which contains the zinc finger domain, might be critical for degradation of ubiquitinated MDM2. It has been shown that the Ring finger domain is required for MDM2 binding to proteasome subunit C8 (36). It is interesting to test whether the L11 binding domain of MDM2 is also required for MDM2 targeting to proteasome or whether L11-MDM2 binding leads to conformational change in the Ring finger domain that impairs the binding of this domain to proteasome subunit.

Despite the above speculations, it is clear that direct binding of L11 to MDM2 is essential for stabilizing MDM2 and inducing p53, as the MDM2 binding-defective deletion mutants were unable to affect MDM2 level and ubiquitination in cells (Fig. 7). Conversely, the MDM2 binding domain-containing deletion of L11 was able to stabilize MDM2 and to enhance its ubiquitination (Fig. 7). Our functional mapping defined the central amino acids 51–108 domain as the important region for regulating MDM2 function. Interestingly, the function of this domain resembles that of the N-terminal domain (amino acids 1–30) of ARF, which is required for ARF inhibitory effect on MDM2 function (41,57). Although sequence comparison between these two domains showed no significant homology (data not shown), it is likely that these two MDM2 binding domains may share structural similarity. Therefore, solving the crystal structure of MDM2-ARF and MDM2-L11 complexes is crucial for better understanding the detailed molecular mechanisms underlying the regulation of MDM2 function by L11 and ARF.

Our results have several important implications. First, L11 differentially regulates the level of ubiquitinated species of p53 and MDM2. L11 decreased ubiquitinated p53 species by inhibiting MDM2-mediated p53 ubiquitination, whereas it increased the ubiquitinated species of MDM2 by blocking proteasome-mediated degradation of ubiquitinated MDM2. Second, our results indicate for the first time that L11 regulates MDM2 through a post-ubiquitination mechanism. Third, because the ultimate inhibition of MDM2 function toward p53 (29–33) by L11 is linked to elevated levels of MDM2 through inhibition of its proteasome-mediated degradation by L11, these results also indicate that proper proteasome-mediated turnover of MDM2 is essential for its E3 ligase activity in cells.

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Figure 1. Regulation of MDM2-mediated p53 and MDM2 ubiquitination by ribosomal proteins L5, L11, L23, and ARF

A, L11 and ARF, but not L5 and L23, increase MDM2-mediated ubiquitinated species of p53. H1299 cells were transfected with combinations of p53 (1 μg), HA-MDM2 (2 μg), V5-ARF (2 μg), FLAG-L5 (2 μg), FLAG-L11 (2 μg), or FLAG-L23 (2 μg) plasmids in the presence of the His-ubiquitin (His-Ub) (2 μg) plasmid as indicated (lanes 1–7). As a control, cells transfected with His-Ub, p53, and HA-MDM2 plasmids were treated with MG132 (20 μM) for 8 h before harvesting (lane 8). The GFP expression vector pEGFP-C1 (0.2 μg) was included in each transfection as an indication of transfection efficiency. The in vivo ubiquitination assay was performed as described under “Materials and Methods.” Ubiquitinated proteins were detected by immunoblot (IB) with the anti-p53 (DO-1) antibody (upper panel). Ubiquitinated p53 (p53-(His-Ub)n) is indicated. The expression of total p53 is shown in the lower panel. B, L11 and ARF, but not L5 and L23, increase the level of ubiquitinated species of MDM2 in cells. The same blot as in A was immunoblotted with anti-HA antibody. Ubiquitinated MDM2
(MDM2-(His-Ub)n) is indicated. The expression of total MDM2 is shown in the lower panel. The asterisk indicates nonspecific anti-HA antibody-reacting bands. C, the cell lysates (50 μg) from the above transfection were immunoblotted with anti-FLAG and anti-V5 antibodies to show the expression of transfected L5, L11, L23, and ARF as indicated on right (upper panel). GFP expression is shown in the lower panel.
FIGURE 2. Regulation of MDM2-mediated p53 and MDM2 ubiquitination by ribosomal proteins L5, L11, L23, and ARF in the presence of MG132

A, L11 and ARF slightly, but L5 and L23 dramatically, inhibit MDM2-mediated p53 ubiquitination in cells. H1299 cells were transfected with combinations of p53 (1 μg), HA-MDM2 (2 μg), V5-ARF (2 μg), FLAG-L5 (2 μg), FLAG-L11 (2 μg), or FLAG-L23 (2 μg) plasmids as well as the His-Ub (2 μg) and pEGFP-C1 plasmids as indicated. The cells were then treated with MG132 (20 μM) for 8 h before harvesting. The in vivo ubiquitination assay was performed. Ubiquitinated proteins were detected by immunoblot (IB) with the anti-p53 (DO-1) antibody (upper panel). Ubiquitinated p53 (p53-(His-Ub)n) is indicated. The expression of total p53 is shown in the lower panel. B, L11 and ARF, but not L5 and L23, increase ubiquitinated species of MDM2 in cells. The similar transfection.
and ubiquitination assay performed as in A was subjected to immunoblot with anti-HA antibody. Ubiquitinated MDM2 (MDM2-(His-Ub)n) is indicated. The expression of total MDM2 is shown in the lower panel. The asterisk indicates non-specific anti-HA antibody-reacting bands. C, the cell lysates (50 μg) from the above transfection were immunoblotted with anti-FLAG and anti-V5 antibodies to show the expression of transfected L5, L11, L23, and ARF as indicated on the right of the upper panel. GFP expression is shown in the lower panel.
FIGURE 3. L11 inhibits 26 S proteasome-mediated degradation of ubiquitinated MDM2 

**A**, in vitro auto-ubiquitination of MDM2. 10 ng of purified FLAG-MDM2 was incubated in the ubiquitination reaction in the presence or absence of E1, UbcH5a (E2), and ubiquitin (Ub). Ubiquitinated MDM2 species (MDM2-(His-Ub)n) were detected by immunoblot using anti-MDM2 antibody. **B**, MDM2 ubiquitination is required for its proteasome degradation. Purified FLAG-MDM2 (10 ng) was incubated with buffer or purified 26 S proteasome, and then the reactions were assayed by anti-MDM2 immunoblot (N20). C, L11 inhibits 26 S proteasome-mediated degradation of ubiquitinated MDM2. In vitro ubiquitinated FLAG-MDM2 (Ub-MDM2) was preincubated with or without His-L11, His-L5, or His-L23. 26 S proteasome (1 µg) or proteasome inhibitor (10 µM MG132, 10 µM lactacystin, 10 µM clastolactacystin-β-lactone)-treated 26 S proteasome was then added. Reactions were quenched in SDS-PAGE sample buffer and analyzed by immunoblot using anti-MDM2 (N20) antibody. A representative experiment was shown. Similar results were obtained in three independent experiments. The *asterisk* indicates nonspecific anti-N20 antibody-reacting bands.
FIGURE 4. L11 stabilizes both endogenous p53 and MDM2 in cells

A, ectopic expression of L11 increases p53 and MDM2 levels in cells. U2OS cells were transfected with FLAG-L11 (3 μg, lane 2) or empty vector (3 μg, lane 1). The cell lysates (50 μg) were immunoblotted (IB) with anti-p53 (DO-1), anti-FLAG, or anti-tubulin antibodies as indicated.

B, ectopic expression of L11 increases the half-life of p53 in cells. U2OS cells were transfected with FLAG-L11 (3 μg, right panels) or empty vector (3 μg, left panels) in the presence of pEGFP-C1 vector (0.1 μg). Forty-eight hours post-transfection the cells were treated with 50 μg/ml cyclohexamide (CHX) and harvested at different time points as indicated. The cell lysates (50 μg) were immunoblotted with anti-p53 (DO-1), anti-FLAG, or anti-GFP antibodies as indicated.

C, the half-life of p53 in cells transfected with FLAG-L11 or an empty vector. The density of p53 in each lane of panel B was quantified against the level of GFP and plotted in a graph.
FIGURE 5. L11 stabilizes MDM2 independent of p53

A, ectopic expression of L11 increases the half-life of MDM2 in the absence of p53. H1299 cells were transfected with HA-MDM2 (1 μg, left panels) or HA-MDM2 plus FLAG-L11 (2 μg, right panels) in the presence of pEGFP-C1 vector (0.1 μg). Forty-eight hours post-transfection, the cells were treated with 50 μg/ml cyclohexamide (CHX) and harvested at different time points as indicated. The clear cell lysates (50 μg) were immunoblotted with anti-HA, anti-FLAG, or anti-GFP antibodies as indicated. The asterisk indicates nonspecific anti-HA antibody-reacting bands (same for below).

B, the half-life of exogenous MDM2 in H1299 cells transfected with FLAG-L11 or an empty vector. The density of MDM2 in each lane of panel A was determined, and the MDM2 levels were normalized against the expression of GFP and plotted in a graph.

C, enhancement of MDM2 protein level by L11 is not due to increased translation. H1299 cells were transfected with HA-MDM2 (1 μg, lane 1) or HA-MDM2 plus FLAG-L11 (2 μg, lane 2) plasmids. The cells were pulse-labeled with 50 μCi/ml [35S]methionine for 15 min. Equal amounts of total proteins were immunoprecipitated (IP) with anti-HA antibody for autoradiography (top panel). The lysates were also directly subjected to immunoblot (IB) using anti-HA or anti-FLAG antibodies (middle and bottom panels). The asterisk indicates nonspecific anti-HA antibody-reacting bands.

D, L11 enhances ubiquitinated MDM2 species in the absence of p53. p53 null H1299 cells were transfected with His-Ub (2 μg), HA-MDM2 (2 μg), FLAG-L11 (2 μg) plasmids as indicated. The cells were harvested after 48 h and subjected to in vivo ubiquitination assays. Ubiquitinated MDM2 was detected by immunoblot with the anti-HA antibody (top panel). The ubiquitinated MDM2 species
(MDM2-(His-Ub)n) are indicated on the right. The cell lysates were also subjected to direct immunoblot with antibodies as indicated on the left of three bottom panels. E, L11 does not increase the levels of p63 in H1299 cells and p53 in p53+/−/mdm2+/− MEF cells. H1299 cells were transfected with p63γ (1 μg) with (lane 1) or without (lane 2) FLAG-L11 (2 μg) in the presence of pEGFP-C1 (0.1 μg) plasmids. Cell lysates were subjected to immunoblot using anti-p63 or anti-FLAG antibodies. p53−/−/mdm2−/− MEF cells were trans-fected with p53 (1 μg, lane 3) or p53 plus FLAG-L11 (2 μg, lane 4) in the presence of pEGFP-C1 (0.1 μg) plasmids. Cell lysates were then used for immunoblot with anti-p53 and anti-FLAG antibodies.
FIGURE 6. The ubiquitin E3 ligase activity of MDM2 is required for L11 to enhance MDM2 ubiquitination

H1299 cells (A) or mouse p53−/−/mdm2−/− MEFs (B) were transfected with wild type (wt) or MDM2C464A mutant (2 μg) in the presence or absence of FLAG-L11 (2 μg) together with His-Ub (2 μg) as indicated. The cell lysates were subjected to ubiquitination assays. The ubiquitinated MDM2 species (MDM2-(His-Ub)n) were detected by immunoblot with the anti-MDM2 (2A10) antibody. The ubiquitinated MDM2 species were shown (top panel). The cell lysates were also subjected to direct immunoblot with 2A10, anti-FLAG, or anti-GFP antibodies as indicated on the three bottom panels.
FIGURE 7. The central domain of L11 binds to MDM2 and is required for L11 to stabilize MDM2, enhance the ubiquitinated species of MDM2, and activate p53.

A, L11 binds to the central region of L11 in cells. H1299 cells were transfected with HA-MDM2 (3 μg) and FLAG-tagged wild-type (wt) or its deletion mutants (3 μg) as indicated. The cell lysates (500 ng) were immunoprecipitated (IP) with the anti-FLAG antibody and immunoblotted with anti-HA or anti-FLAG antibodies (top panel). Cell lysates (50 μg) were also directly subjected to immunoblot with anti-HA and anti-FLAG antibodies as indicated on the left of each bottom panel. B, diagram of the FLAG-tagged wild-type L11 and its deletion mutants. The co-immunoprecipitation results determined in panel A are shown on the right. A plus symbol indicates binding, and a minus symbol indicates lack of binding. C, the central region-containing deletion mutants of L11 (L111–108) can partially enhance the ubiquitinated species of MDM2 in the absence of p53. H1299 cells were transfected with His-Ub (2 μg), HA-MDM2 (2 μg) with FLAG-tagged wild type, or its deletion mutants (2 μg) as indicated. The cells lysates were subjected to ubiquitination assays. The ubiquitinated MDM2 species (MDM2-(His-Ub)n) were detected by immunoblot with the anti-HA antibody. The ubiquitinated MDM2 species are shown (top panel). The cell lysates were also subjected to direct immunoblot (IB) with anti-HA, anti-FLAG, or anti-GFP antibodies as indicated on the left of the three bottom panels. D, wild-type L11 and L111–108 mutant, but not other deletion mutants, stabilize and activate endogenous p53 in cells. U2OS cells were transfected with wt L11 or its deletion mutants as indicated. Forty-eight hours post-transfection, cells were harvested and subjected to immunoblot with anti-p53 (DO-1), anti-p21, anti-MDM2 (2A10), or anti-tubulin antibodies as indicated on the left of each panel.