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MDM2 and MDMX can interact differently with ARF and members of the p53 family

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Abstract Members of the p53 family of transcription factors have essential roles in tumor suppression and in development. MDM2 is an essential regulator of p53 that can inhibit the transcriptional activity of p53, shuttle p53 out of the nucleus, and target p53 for ubiquitination-mediated degradation. Little is known about the interaction and selectivity of different members of the p53 family (p53, p63, and p73) and the MDM2 family (MDM2 and MDMX). Here we show that the transcriptional activities of p53 and p73, but not that of p63, were inhibited by both MDM2 and MDMX. Consistent with these, we found that MDMX can physically interact with p53 and p73, but not with p63. Moreover, ectopically expressed MDM2 and MDMX could induce alterations in the subcellular localization of p73, but did not affect the subcellular localization of p53 and p63. Finally, we demonstrate that while ARF can interact with MDM2 and inhibit the regulation of p53 by MDM2, no interaction was found between ARF and MDMX. These data reveal that significant differences and selectivity exist between the regulation of different members of the p53 family by MDM2 and MDMX.

Key words: ARF; MDM2; MDMX; p53; p63; p73; Tumor suppressor

1. Introduction

Loss of the p53 tumor suppressor function is one of the most common steps in tumorigenesis of many types of cancer [1]. The functions of p53 are primarily mediated through its transcriptional activation of genes that are involved in the regulation of cell cycle checkpoints and apoptosis [2]. The activity of p53 is mainly controlled at the levels of transcriptional activity and protein stability. One of the transcriptional targets of p53, MDM2, can regulate both the transcriptional activity and the half life of p53 in a negative feedback loop [3]. MDM2 can bind to the N-terminal transactivation domain of p53 and inhibit p53-mediated transcription [4–6]. Interaction between MDM2 and p53 also results in the shuttling of p53 out of the nucleus by the virtue of the nuclear exporting signal in MDM2 [7–10]. Furthermore, MDM2 reduces the level of p53 by targeting p53 for ubiquitin-mediated proteolysis [11,12] through the ubiquitin ligase function of MDM2 [13,14].

After DNA damage, phosphorylation of p53 at N-terminal serine residues by protein kinases like ATM, ATR or DNA-PK inhibits the binding of MDM2 to p53, therefore leading to an increase in p53 level, activity, and nuclear localization [15–17]. MDM2 is overexpressed in a variety of tumors, which would eliminate p53 responses after DNA damage and other stresses [18]. The inhibition and degradation of p53 by MDM2 are subjected to negative regulation by ARF [19,20], which sequesters MDM2 to the nucleolus [21,22].

A protein that is related in sequence to MDM2, MDMX, has been identified in mammalian cells [23,24]. Similarly to MDM2, there are also several forms of MDMX transcripts [24,25] and MDMX gene expression remains constitutive during cell proliferation and differentiation [26]. Unlike MDM2, however, the mRNA and protein of MDMX are not induced by UV irradiation [24,26]. The p53 binding domain and the RING finger motif of MDM2 are conserved in MDMX. Accordingly, MDMX can bind to p53 and inhibits its transcriptional activity [24,27]. However, unlike MDM2, MDMX is not able to facilitate the degradation of p53 [28]. MDMX can interact with MDM2 through the C-terminal RING finger motifs of the two proteins [29], and MDM2 is stabilized when it binds to MDMX [30].

Several proteins that share a high degree of similarity to p53 have been identified [31]. These include p73 and its alternatively spliced forms [32–34], and p63 and its various variants (two of them were also cloned as p51A and p51B) [35–37]. Like p53, p73 can also bind to MDM2. But in contrast to p53, binding of MDM2 to p73 does not target p73 for degradation [38–42]. Recently, it was found that like p53, p73 is also induced by DNA damage in a c-Abl-dependent manner [43–45]. The induction of p73 is mediated through the interaction between the SH3 domain of c-Abl and the C-terminal homo-oligomerization domain of p73, and the phosphorylation of p73 on Tyr99 by c-Abl.

Mice with disrupted p73 have neurological, pheromonal and inflammatory defects [34]. Unlike $p53^{-/-}$ mice, however, $p73^{-/-}$ mice do not develop tumor spontaneously. The p63 gene product appears to have an important function in development. Disruption of p63 in mice indicates that p63 is essential for limb and epithelial development [46,47], and heterozy-

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gous germline mutations of p63 genes have been found in the human EEC syndrome [48].

Little is known about the selectivity in the interaction between members of the MDM2 family (MDM2 and MDMX) and the p53 family (p53, p63, and p73). Here we have explored whether p53, p63, and p73 can be regulated by MDM2 and MDMX, and whether MDM2 and MDMX can be regulated by ARF. We demonstrate that p53 and p73 are inhibited by both MDM2 and MDMX, but p63 does not interact with MDM2 or MDMX. MDM2 and MDMX can affect the subcellular localization of p73, but little effect was observed with p53 and p63. Finally, we found that ARF can interact with MDM2 but not with MDMX.

2. Materials and methods

2.1. DNA constructs

Human p53 in pRcCMV was a gift from Dr. Arnold Levine (Princeton University). Human p63α (p51B) in pRcCMV was a gift from Dr. Shuntaro Ikawa (Tohoku University). FLAG-p63 in pUHD-P1 was constructed by amplifying the p63 cDNA by PCR using the Sp6 primer and the oligonucleotide 5′-CCACCATGGCCCAGAGCACACAG-3′; the PCR product was cut with *Nco*I (partial digest) and *Xba*I, and ligated into *Nco*I–*Xba*I cut pUHD-P1. Constructs of hemagglutinin (HA)-tagged simian p73 in pcDNA3 were from Dr. Daniel Caput (Sanofi Recherche, Labege, France). FLAG-p73α(NΔ250) in pUHD-P1 and FLAG-p73β(NΔ250) in pUHD-P1 were constructed by putting the *Eco*RI–*Xba*I fragment of HA-p73α in pcDNA3 or HA-p73β in pcDNA3, respectively, into *Eco*RI and *Xba*I cut pUHD-P1. HA-p73α in pUHD-P1 and HA-p73β in pUHD-P1 were constructed by putting the *Sac*II–*Eco*RI of HA-p73α in pcDNA3 or HA-p73β in pcDNA3, into *Sac*II–*Eco*RI cut FLAG-p73α(NΔ250) in pUHD-P1 or FLAG-p73β(NΔ250) in pUHD-P1, respectively. MDM2 in pCMV, and $p21^{CIP1/WAF1}$ promoter-luciferase reporter construct were gifts from Dr. Bert Vogelstein (Howard Hughes Medical Institute, Johns Hopkins Oncology Center). The β-galactosidase construct was a gift from Dr. Yan Chen (The Salk Institute). GST-MDM2 in pCAGGS and GST-MDMX in pCAGGS were as described [40]. FLAG-MDMX in pUHD-P1 was constructed by amplification of the MDMX cDNA in pBluescript with 5′-GTCCCATGGCATCATTTTCCACCT-3′ and T7 primer, the *Nco*I–*Bam*HI fragment was then put into pUHD-P1. Human ARF in pBluescript KS+ was a gift from Dr. Gordon Peters (ICRF, London). The ARF coding region was amplified by PCR with 5′-GACCATGGTGCGCAGGTTCTTGGT-3′ and T3 primer, cut with *Nco*I–*Xho*I and put into pGEX-KG. The *Nco*I–*Eco*RI fragment of GST-ARF in pGEX-KG was put into *Nco*I–*Eco*RI cut pUHD-P1 to give FLAG-ARF in pUHD-P1.

2.2. Cell culture

H1299 cells (human non-small cell lung carcinoma cells) were obtained from the American Type Culture Collection (Rockville, MD, USA). HtTA1 cells are HeLa cells (human cervical carcinoma cells) with stable integration of pUHD15-1, which expresses the tTA tetracycline repressor chimera [49]. HtTA1 cells can express genes cloned into the pUHD-P1 and pLINX vectors in the absence of deoxycycline. Cells were grown in DMEM supplemented with 10% v/v calf serum (for HeLa cells) or 10% v/v fetal bovine serum (Gibco-BRL) in a humidified incubator at 37°C with 5% CO_2. Semi-confluent cells were transiently transfected with the calcium phosphate precipitation method [50]. Unless stated otherwise, 2 μg of p53, p73, and p63 constructs, and 10 μg of other plasmids were used to transfect 10 cm plates. The total amount of DNA for each transfection was adjusted to the same level using vectors with the same promoter. Cell-free extracts were prepared as described previously [51]. The protein concentration of cell lysates was measured with bicinchoninic acid protein assay system (Pierce) using bovine serum albumin as a standard.

2.3. Luciferase and β-galactosidase assays

Luciferase assays and β-galactosidase assays were performed exactly as described [52]. The luciferase activities were normalized with the β-galactosidase activities.

2.4. Glutathione (GSH)–agarose binding

GST fusion proteins were recovered with 15 μl of GSH-agarose in 250 μl of bead buffer [53]. After incubation at 4°C with end-to-end rotation for 45 min, the beads were washed five times with 250 μl of bead buffer. The samples were then dissolved in 30 μl of sodium dodecyl sulfate (SDS) sample buffer, and the bound proteins were detected by SDS–PAGE followed by immunoblotting.

2.5. Antibodies and immunological methods

Rabbit anti-GST antibodies were gifts from Dr. Julian Gannon and Tim Hunt (ICRF, South Mimms). Monoclonal antibody 2A10 against MDM2 was a gift from Dr. Arnold Levine (Princeton University). Monoclonal antibody M2 against FLAG tag was obtained from Eastman Kodak. Rabbit anti-FLAG polyclonal antibodies were raised against the FLAG peptide (DYKDDDDK). Monoclonal antibody DO-1 against p53 was obtained from Santa Cruz (sc-126). Monoclonal antibody 12CA5 against HA tag was from Roche Molecular Biochemicals. Immunoblottings were performed as described previously [53]. Immunoprecipitations were performed as described previously [54]. Indirect immunofluorescence staining and microscopy were as described [52]. The images were captured with a color cooled CCD camera (CoolSnap, Photometrics) with a Macintosh computer.

3. Results

3.1. MDM2 and MDMX do not inhibit the transcriptional activity p63

We set out to explore the possibility whether different members of the p53 family (p53, p63, and p73) are regulated similarly by MDM2 and the related protein MDMX. The p53-null lung carcinoma H1299 cells were co-transfected with various constructs and a reporter construct containing the $p21^{CIP1/WAF1}$ promoter placed upstream of luciferase. The luciferase activity was assayed and normalized with the activity of β-galactosidase expressed from a co-transfected plasmid. We found that p63α could induce the transcription of the $p21^{CIP1/WAF1}$ promoter, and the transcriptional activity of p63α was affected by neither MDM2 nor MDMX (Fig. 1A). To confirm that the MDM2 and MDMX used were functional in our system, similar experiments were preformed with p53 and p73. As expected, transcription from the $p21^{CIP1/WAF1}$ promoter was strongly induced in the presence of p53 (Fig. 1B). Co-expression with MDM2 reduced the transcriptional activity of p53 to background level (lane 3). As a control, an N-terminal truncation mutant of MDM2(NΔ297) that does not interact with p53 has no effect on the transactivation activity of p53 (lane 4). In agreement with previous reports, co-expression with MDMX decreased the transcriptional activity of p53 (lane 5). Neither MDM2 nor MDMX alone activated the transcription from the $p21^{CIP1/WAF1}$ promoter (lanes 6 and 7). Fig. 1C,D shows that the $p21^{CIP1/WAF1}$ promoter was transactivated by p73α and the alternatively spliced p73β, but not by a transcriptional-inactive point mutant R292H. Both MDM2 and MDMX could inhibit the transcriptional activity of p73α and p73β. Taken together, these data imply that MDM2 and MDMX can inhibit the transcriptional activities of p53 and p73, but not that of the related protein p63.

3.2. MDMX physically interacts with p53 and p73, but not p63

We next looked at whether MDMX can directly bind to p53, p63, and p73. A GST-tagged MDMX was co-expressed with p53, FLAG-tagged p63α, or HA-tagged p73α in mammalian cells. Cell extracts were prepared and the GST-MDMX and associated proteins were precipitated with GSH-agarose. Proteins that associate with MDMX were de-

tected by immunoblotting with specific antibodies. Fig. 2 shows that both p53 and p73α were efficiently co-precipitated with MDMX (lane 2). As a control, neither p53 nor p73 was precipitated with control agarose beads (lane 3). In striking contrast to p53 and p73, p63α was not co-precipitated with MDMX. Similarly, we found that GST-MDM2 could bind to p53 and p73, but not to p63α ([40] and data not shown). These results indicate that MDM2 and MDMX can physically interact with p53 and p73α but not with p63α.

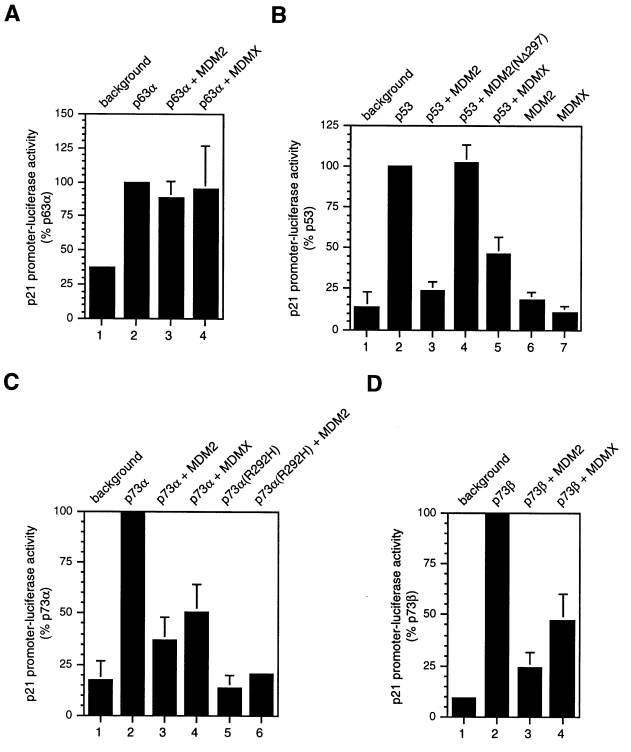

Fig. 1. MDM2 and MDMX inhibit the transcriptional activity of p53, p73, but not p63. (A) p63α is not inhibited by MDM2 or MDMX. Control plasmids (lane 1), plasmids expressing p63α (lane 2), p63α and MDM2 (lane 3), or p63α and MDMX (lane 4) were transfected into H1299 cells. All samples were co-transfected with plasmids expressing the $p21^{CIP1/WAF1}$ promoter-luciferase reporter (0.2 μg) and the β-galactosidase (0.5 μg). Cell extracts were prepared at 36 h after transfection and the luciferase activity was determined as described in Section 2. (B) The transcriptional activity of p53 can be inhibited by MDM2 and MDMX. H1299 cells were transfected with control plasmids (lane 1), plasmids expressing p53 (lane 2), p53 and MDM2 (lane 3), p53 and MDM2(NΔ297) (lane 4), p53 and MDMX (lane 5), MDM2 (lane 6), or MDMX (lane 7). The $p21^{CIP1/WAF1}$ promoter luciferase activities were determined as described in Section 2. The average of three independent experiments and their standard deviations are shown. (C) Inhibition of the transactivation activities of p73α by MDM2 and MDMX. H1299 cells were transfected with control plasmids (lane 1), plasmids expressing p73α (lane 2), p73α and MDM2 (lane 3), p73α and MDMX (lane 4), p73α(R292H) (lane 5), or p73α(R292H) and MDM2 (lane 6). The $p21^{CIP1/WAF1}$ promoter luciferase activities were determined as described in Section 2. The average of three independent experiments and their standard deviations are shown. (D) Inhibition of the transactivation activities of p73β by MDM2 and MDMX. Control plasmids (lane 1), plasmids expressing p73β (lane 2), p73β and MDM2 (lane 3), or p73β and MDMX (lane 4) were transfected into H1299 cells, and the $p21^{CIP1/WAF1}$ promoter luciferase activities were determined as in (A).

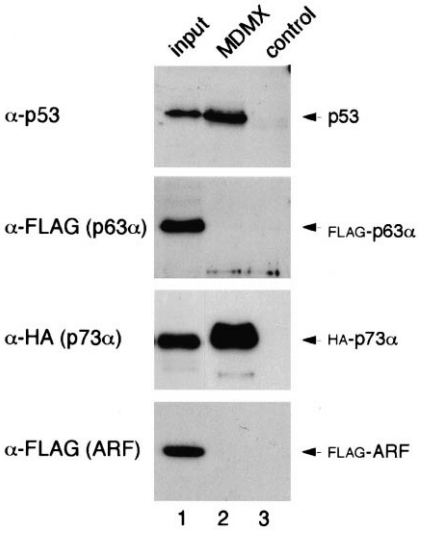

Fig. 2. MDMX interacts with p53 and p73, but not p63 and ARF. HtTA1 cells were co-transfected with plasmids expressing GST-MDMX and p53 (upper panel), FLAG-p63α (second panel), HA-p73α (third panel), or FLAG-ARF (bottom panel). Cell extracts were prepared and 100 μg of the extracts was incubated with GSH-agarose (lane 2) or control agarose beads (lane 3) as described in Section 2. The beads were washed, dissolved in SDS sample buffer, and the samples were immunoblotted with monoclonal antibodies against p53, FLAG, or HA as indicated. The total cell lysates (10 μg) were loaded in lane 1 to indicate the efficiency of the binding.

3.3. *Ectopically expressed MDMX can alter the subcellular localization of p73, but not p53 and p63*

Given that MDMX can interact with p53 and p73, we next investigated whether MDMX could affect the subcellular localization of these proteins. MDMX was co-expressed with p53, p63α, or p73α in the cervical carcinoma HtTA1 cells, and the localization of the proteins was analyzed by indirect immunofluorescence microscopy (Figs. 3 and 4). Ectopically expressed p53, p63α, and p73α were localized to the nucleus (data not shown). Co-expression of MDMX or MDM2 with p73α induced dramatic changes in the localization of p73α. Fig. 3 shows that co-expression of MDM2 induced a redistribution of p73α from the nucleus to paranuclear regions. When MDMX was co-expressed with p73α, p73α was redistributed to the cytoplasm. As a control, we investigated the subcellular localization of an N-terminal truncation mutant of p73α(NΔ250), which does not interact with MDM2 or MDMX [40]. The subcellular localization of p73α(NΔ250) was not affected by co-expression of MDMX (Fig. 3B) or MDM2 (data not shown). In contrast to p73α, the nuclear localization of p53 and p63α was not affected by MDMX (Fig. 4). Fig. 4 shows that even in cells with high expression of both p63α and MDMX, p63α was localized to the nucleus whereas MDMX was localized to the cytoplasm. The subcellular localization of p63α was similarly not affected by MDM2 (data not shown). The localization of p53 in the presence of MDM2 was not shown since MDM2 targeted p53 for degradation and the immunofluorescent signal was very weak.

3.4. *ARF interacts with MDM2 but not with MDMX*

ARF can interact with MDM2 and modulate the regulation of p53 by MDM2 [21,22]. We next investigated whether ARF can also interact with MDMX. Fig. 2 shows that MDMX did

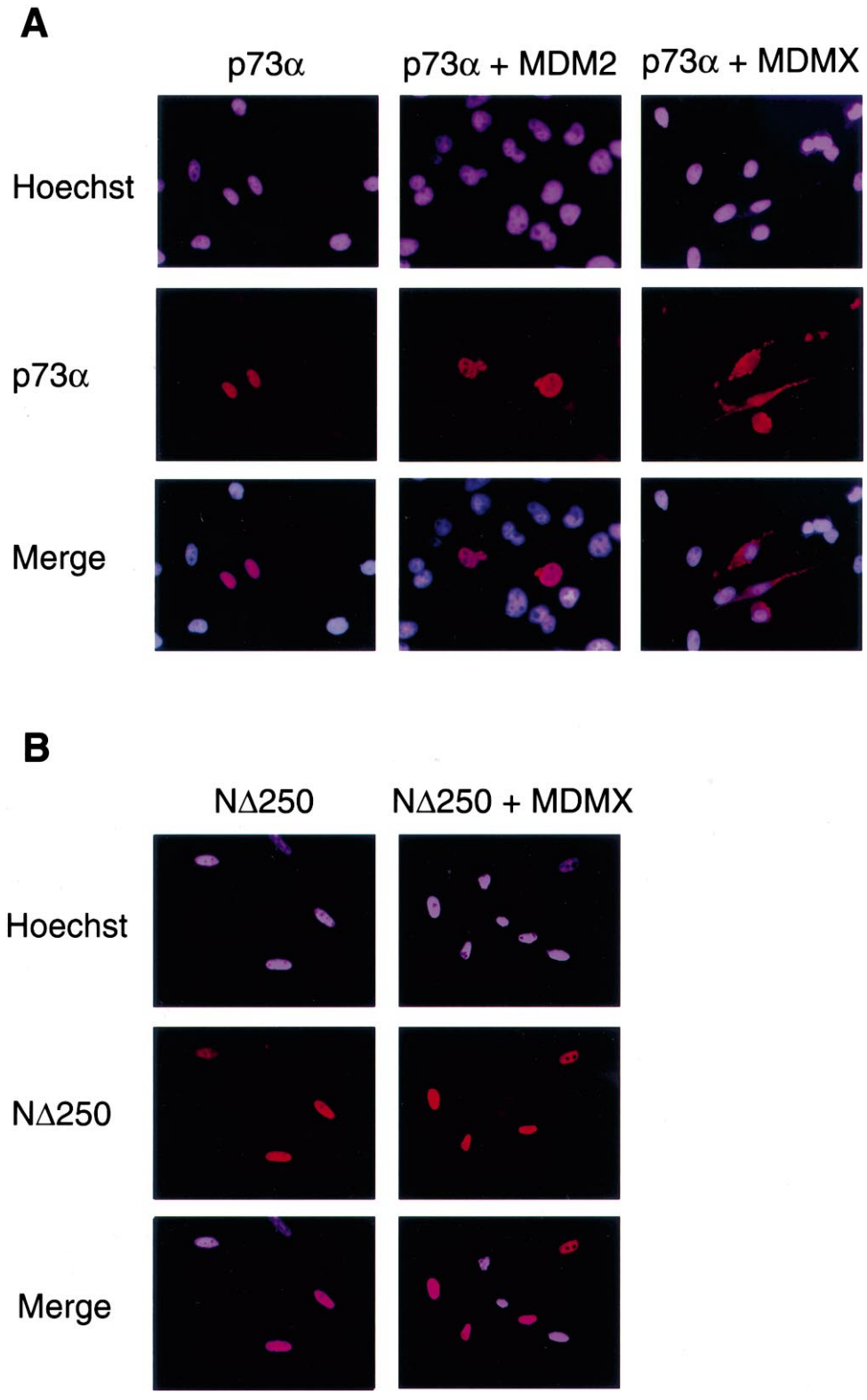

Fig. 3. MDM2 and MDMX alter the localization of p73α. (A) HtTA1 cells were transfected with HA-p73α (first column), HA-p73α and GST-MDM2 (second column), or HA-p73α and GST-MDMX (third column). The cells were fixed and processed for indirect immunofluorescence microscopy as described in Section 2, and representative images are shown. HA-tagged p73α was detected by anti-HA monoclonal antibody (12CA5), followed by TRITC-conjugated secondary antibody (red). Nuclei were stained with Hoechst 33258 dye (blue). The merged images are shown in the bottom panels. (B) HtTA1 cells were transfected with the FLAG-tagged NΔ250 mutant of p73α (first column), or p73α(NΔ250) and GST-MDMX (second column). FLAG-tagged p73α(NΔ250) was detected by anti-FLAG monoclonal antibody (M2) followed by TRITC-conjugated secondary antibody (red). Nuclei were stained with Hoechst 33258 dye (blue). The merged images are shown in the bottom panels.

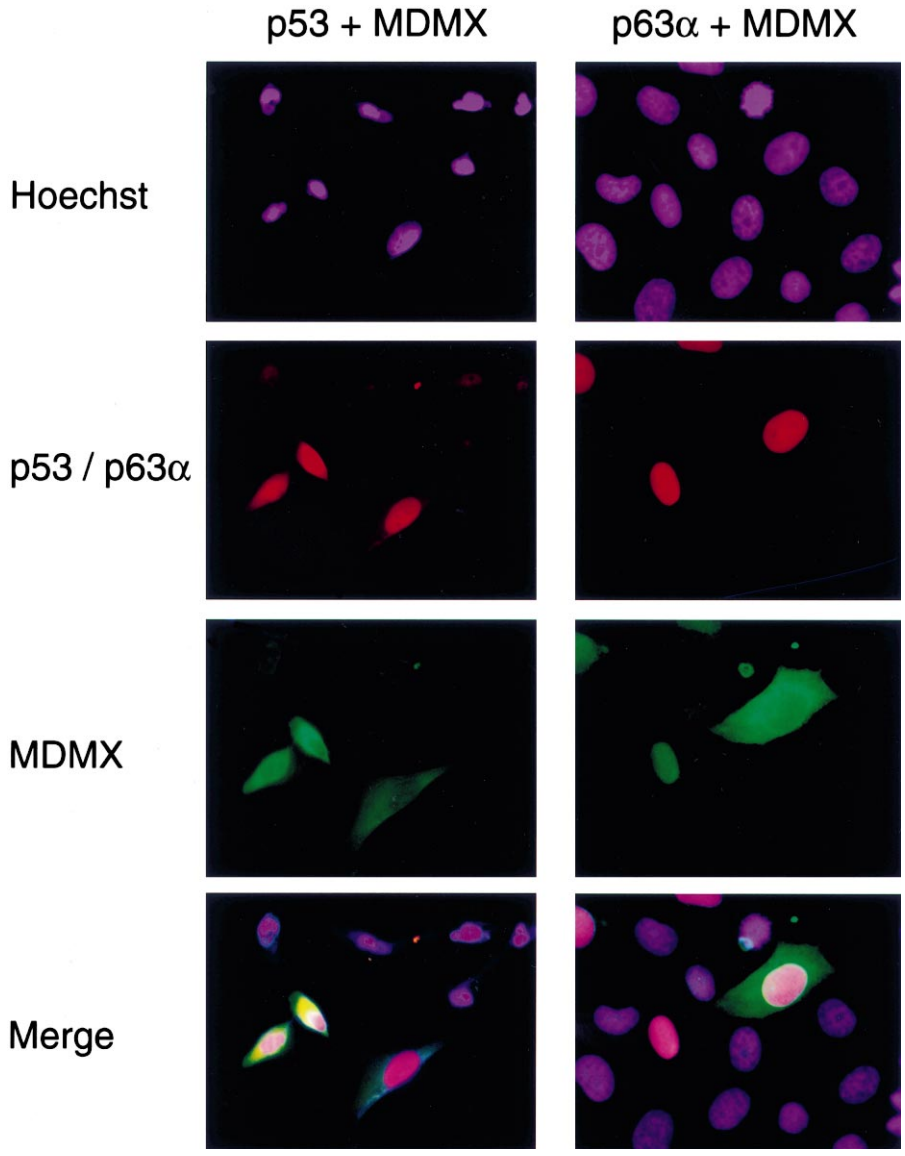

Fig. 4. MDMX does not affect the localization of p53 and p63α. HtTA1 cells were transfected with p53 and GST-MDMX (first column), or FLAG-p63α and GST-MDMX (second column). The cells were fixed and processed for indirect immunofluorescence microscopy as described in Section 2, and representative images are shown. FLAG-p63α and p53 were detected by monoclonal antibodies M2 and DO-1, respectively, followed by TRITC-conjugated secondary antibody (red); GST-MDMX was detected by rabbit anti-GST antibodies, followed by FITC-conjugated secondary antibody (green). Nuclei were stained with Hoechst 33258 dye (blue). The merged images are shown in the bottom panels.

not form a stable complex with ARF (bottom panel, lane 2). To investigate the possible interaction between MDMX and ARF in more detail, MDM2 or MDMX was co-expressed with ARF and p53 in mammalian cells; cell extracts were then prepared and MDM2/MDMX were precipitated to examine the associated proteins (Fig. 5). As shown above, p53 could be co-precipitated with both MDM2 and MDMX (lanes 10 and 11, 13 and 14). MDM2 binds to ARF irrespective of the presence or absence of p53 (middle panel, lanes 12 and 13), confirming that the ARF used was functional. In contrast, MDMX did not form a stable complex with ARF even when both proteins were overexpressed (lanes 9 and 10). In agreement with the known function of ARF, the total level of p53 was stabilized when co-expressed with ARF (lanes 3 and 4 and 6 and 7). It is interesting that although ARF did not interact with MDMX, expression of ARF stabilized p53 that was co-expressed with MDMX. One explanation is that the expressed ARF could interact with the endogenous MDM2 and stabilized p53. The control blot for tubulin (bottom panel) indicates that a similar amount of extracts was loaded in each lane. Taken together, these data indicate that ARF can interact with MDM2 but not with the related MDMX.

4. Discussion

Here we show that members of the p53 family (p53, p63, and p73) can be regulated differently by MDM2 and MDMX. Furthermore, there are several aspects that distinguish MDM2 and MDMX in their regulation of these p53-related proteins. Table 1 summarizes the known interaction between members of the p53 family and the MDM2 family. Both MDM2 and MDMX could form complexes with p53 and p73, but not with p63 (Fig. 2). In agreement with their bind-

Table 1
Summary of the interaction between p53, p63α, p73α, MDM2, MDMX, and ARF

	Binding		Inactivation of transcription		Promotion of degradation		Change in subcellular localization	
	MDM2	MDMX	MDM2	MDMX	MDM2	MDMX	MDM2	MDMX
p53	+	+	+	+	+	−	+	−
p63α	−	−	−	−	−	−	−	−
p73α	+	+	+	+	−	−	+	+
ARF	+	−	NA	NA	ND	ND	ND	ND

ing capability, the transcriptional activities of p53 and p73, but not that of p63, were inhibited by MDM2 and MDMX (Fig. 1). Given that p53, p73, and p63 can activate similar promoters, the differential regulation of these related proteins by MDM2 and MDMX may provide a mechanism to regulate p53-responsive promoters. It is conceivable that at a constant level of MDM2, transcription from the p53-responsive promoters can be modulated by varying the ratios between p63 and p53.

Why do p53 and p73, but not p63, interact with MDM2/MDMX? Given that the N-terminal region of p53, which is believed to be involved in binding to MDM2, is equally similar to both p73 and p63, it is thus surprising that p63 could not bind to MDM2 or MDMX. One possible difference between the N-terminal sequences that may explain why p63 does not bind to MDM2/MDMX is the extra eight residues found in p63 in comparison to p53 and p73. It was reported that apart from the N-terminus, the C-terminal region of p53 also plays a role in MDM2-directed degradation of p53 [55]. Hence the variation of the C-terminal region between p53/p73 and p63 may also explain why p63 does not interact with MDM2/MDMX.

MDM2 and MDMX are likely to have distinct functions in the cell, since it has been shown that MDM2, but not MDMX, is a transcriptional target of the p53. We found that ARF could bind to MDM2 but not to MDMX (Figs. 2 and 5). Since MDM2 interacts with both p53 and p73, it is likely that ARF can regulate the activity of both p53 and p73 through MDM2 but not through MDMX. These results imply that the relative level of MDM2 and MDMX in the cell may determine the p53/p73 response to ARF. In this connection, it would be interesting to identify the region of MDM2 that interacts with ARF to understand why MDMX does not bind to ARF. These data also suggest that overexpression of MDMX can be a mechanism for some cells to escape regulation by ARF.

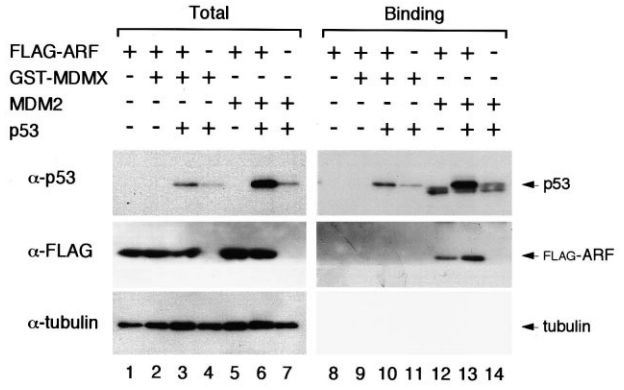

Fig. 5. MDM2 but not MDMX interacts with ARF. HtTA1 cells were transfected with plasmids expressing FLAG-ARF (lanes 1–3, 5, 6, 8–10, 12 and 13), GST-MDMX (lanes 2–4 and 9–11), MDM2 (lanes 5–7 and 12–14), and p53 (lanes 3, 4, 6, 7, 10, 11, 13, 14). Total cell extracts (10 μg) were loaded in lanes 1–7. Cell extracts (100 μg) were incubated with GSH-agarose (lanes 8–11) or immunoprecipitated with the anti-MDM2 monoclonal antibody 2A10 (lanes 12–14). The samples were loaded onto 12.5% SDS–PAGE and immunoblotted with antibodies against p53 (upper panels), FLAG (middle panels), or tubulin (bottom panel, for lanes 1–7 only). The lower bands in the MDM2 immunoprecipitates (lanes 12–14) are the IgG heavy chains.

MDM2 and MDMX appear to affect the subcellular localization of p73 differently (Fig. 3). Ectopically expressed MDMX was predominantly localized to the cytoplasm [25,52] (Fig. 4). Similar data were obtained using FLAG-tagged MDMX instead of GST-tagged MDMX, or using the human lung carcinoma H1299 cells (unpublished data). The predominantly nuclear staining of p53 and p63 was not affected by the co-expression of MDMX (Fig. 4) or MDM2 (unpublished data). The lack of effect of MDM2 and MDMX on the localization of p63α is not surprising since the proteins do not associate with each other. Little p53 was detected when co-expressed with MDM2. It is possible that the p53 that is redistributed to the cytoplasm by MDM2 is rapidly degraded by the proteasome. In contrast, p73 can be readily detected in the cytoplasm because p73 is not targeted for rapid degradation by MDM2. Staining of p53 was detected in cells that co-expressed MDMX because MDMX was shown to be not able to facilitate the degradation of p53 [28]. The rapid shuttling of MDM2 between the cytoplasm and the nucleus is believed to be important for the regulation of p53 by MDM2 [9,10]. It is interesting that both the nuclear localization sequence and the NES of MDM2 are missing in MDMX. Hence, it is possible that MDMX in the cytoplasm can bind to the shuttling p73 and retain the p73 in the cytoplasm.

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