



Different effects of p14ARF on the levels of ubiquitinated p53 and Mdm2 *in vivo*

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Mdm2 has been shown to promote its own ubiquitination and the ubiquitination of the p53 tumour suppressor by virtue of its E3 ubiquitin ligase activity. This modification targets Mdm2 and p53 for degradation by the proteasome. The p14ARF tumour suppressor has been shown to inhibit degradation of p53 mediated by Mdm2. Several models have been proposed to explain this effect of p14ARF. Here we have compared the effects of p14ARF overexpression on the *in vivo* ubiquitination of p53 and Mdm2. We report that the inhibition of the Mdm2-mediated degradation of p53 by p14ARF is associated with a decrease in the proportion of ubiquitinated p53. The levels of polyubiquitinated p53 decreased preferentially compared to monoubiquitinated species. p14ARF overexpression increased the levels of Mdm2 but it did not reduce the overall levels of ubiquitinated Mdm2 *in vivo*. This is unexpected because p14ARF has been reported to inhibit the ubiquitination of Mdm2 *in vitro*. In addition we show that like p14ARF, the proteasome inhibitor MG132 can promote the accumulation of Mdm2 in the nucleolus and that this can occur in the absence of p14ARF expression. We also show that the mutation of the nucleolar localization signal of Mdm2 does not impair the overall ubiquitination of Mdm2 but is necessary for the effective polyubiquitination of p53. These studies reveal important differences in the regulation of the stability of p53 and of Mdm2. *Oncogene* (2001) 20, 4972–4983.

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Introduction

Degradation of proteins through the ubiquitin system involves conjugation of multiple ubiquitin molecules to the target protein and degradation of the ubiquitinated substrate by the 26S proteasome. Ubiquitin conjugation is mediated by the sequential action of three enzymes, namely E1 (ubiquitin-

activating enzyme), E2 (ubiquitin-conjugating enzyme) and E3 (ubiquitin-protein ligase). E3s are the proteins that bind to target substrates, either directly or indirectly via an adapter molecule, and facilitate transfer of ubiquitin to the substrate. E3s have a limited range of substrates and provide the specificity to the system. The conjugation of many ubiquitin molecules to the substrate, in the form of a polymeric chain targets proteins for destruction (Ciechanover, 1998). Tetra-ubiquitin was demonstrated to be the minimum signal for efficient proteasomal degradation (Thrower *et al.*, 1999).

In normal non-stressed cells the level of wild-type p53 is maintained at a low level due to its very short half-life (5–20 min) (Midgley *et al.*, 1995; Maltzman and Czyzyk, 1984). In response to different stress stimuli, including DNA damage and oncogenic signals p53 protein is stabilized and its levels are increased. This results in the induction of p53 transcriptional activity and consequent expression of genes whose products cause cell cycle arrest or induction of apoptosis (Oren, 1999; Levine, 1997; Ko and Prives, 1996). The Mdm2 oncoprotein has been shown to play a major role in the regulation of p53's stability. Interaction of p53 with Mdm2 results in a dramatic reduction of the p53 levels *in vivo* (Haupt *et al.*, 1997; Kubbutat *et al.*, 1997; Bottger *et al.*, 1997). This effect was shown to be due to the ability of Mdm2 to function as an E3 ubiquitin ligase for p53 (Honda *et al.*, 1997; Honda and Yasuda, 2000; Fang *et al.*, 2000). On the other hand, Mdm2 expression is induced by p53's transcriptional activity. In this way, p53 regulates its own stability by a negative feedback loop (Lane and Hall, 1997).

The INK4 α /p14ARF locus encodes two proteins, designated p16INK4 α and p14ARF, by using alternative reading frames. p14ARF was shown to directly interact with Mdm2 and to prevent p53 degradation. This results in the accumulation of p53 and the induction of p53 dependent cell cycle arrest and/or apoptosis (Pomerantz *et al.*, 1998; Zhang *et al.*, 1998). Further studies demonstrated that p14ARF is induced by overexpression of E2F and oncogenes such as E1A, Myc and Ras (reviewed in Vousden, 2000). This ability of p14ARF expression to respond to oncogenic signals and to induce p53 function is thought to be important in the role of p14ARF as a tumour suppressor.

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A number of mechanisms have been reported to be involved in p14ARF-mediated inhibition of the Mdm2-dependent degradation of p53. *In vitro* biochemical studies indicate that p14ARF inhibits the ubiquitin ligase activity of Mdm2 (Honda and Yasuda, 1999; Midgley *et al.*, 2000). In addition, p14ARF has been reported to sequester the p53-Mdm2 complex in discrete subnuclear compartments and to inhibit the nuclear export of the complex, a step thought to be essential for p53 degradation (Zhang and Xiong, 1999). According to Tao and Levine (1999) this export step could occur via the nucleolus. Since the ubiquitination of p53 has been described to be necessary to promote the export of p53 from the nucleus to the cytoplasm (Boyd *et al.*, 2000; Geyer *et al.*, 2000), inhibition of the ubiquitination of p53 by p14ARF could inhibit the export of p53 from the nucleus to the cytoplasm. In another study, p14ARF was proposed to sequester Mdm2 in the nucleolus, preventing the interaction of p53 with Mdm2 and allowing the accumulation of p53 in the nucleoplasm (Weber *et al.*, 1999).

Although the inhibitory effect of p14ARF on the ubiquitination of Mdm2 and on the Mdm2-dependent ubiquitination of p53 has been shown *in vitro*, the effect of p14ARF on the levels of the ubiquitin-conjugates of these proteins in cells has not been reported. Using a quantitative *in vivo* ubiquitination assay, here we show that the inhibition of Mdm2-mediated degradation of p53 by p14ARF is associated with a decrease in the levels of polyubiquitinated p53. Whether p14ARF increases the levels of Mdm2 has been a matter of controversy. Ectopically expressed p14ARF was shown to decrease the levels and half life of exogenous hMdm2 in HeLa cells (Zhang *et al.*, 1998). In another report, p14ARF overexpression was shown to increase the levels of hMdm2 in U2OS cells, which express endogenous wild-type p53 (Stott *et al.*, 1998). Here we show that p14ARF overexpression, like proteasome inhibitors (An *et al.*, 1998), increases the levels of the Mdm2 protein in the absence of p53. Interestingly, this increase in the levels of Mdm2 by p14ARF occurs without an effect on the levels of ubiquitinated Mdm2. This is in striking contrast to the inhibitory effect of p14ARF on Mdm2's ubiquitination *in vitro*. The proteasome inhibitor MG132 and p14ARF are also similar in their ability to induce the localization of Mdm2 in the nucleolus. We also analyse whether the nucleolar localization of Mdm2 is necessary for the ubiquitination or degradation of Mdm2 and for the conjugation of p53 to ubiquitin.

Results

A quantitative in vivo assay for Mdm2-mediated ubiquitination of p53

In order to study Mdm2-mediated ubiquitination of p53 *in vivo* we developed a p53 ubiquitination assay

based on the protocol originally described by Treier *et al.* (1994). We chose to use the H1299 lung carcinoma cell line for the following reasons. First, it is very efficiently transfected, which permits easy detection of proteins linked to ubiquitin. Second, it is deficient for p53 expression (Wang *et al.*, 1998) and therefore, the levels of endogenous hMdm2 are low. H1299 cells were transfected with constructs expressing wild-type human p53, murine Mdm2 and a His₆-tagged version of ubiquitin. Thirty-six hours post transfection cells were lysed in 6 M guanidinium-HCl containing buffer and His₆-ubiquitinated proteins were purified using Ni²⁺-agarose beads as described in Materials and methods. Denaturing conditions were used in order to prevent deubiquitination of protein-ubiquitin conjugates as well as any non-covalent protein-protein interactions.

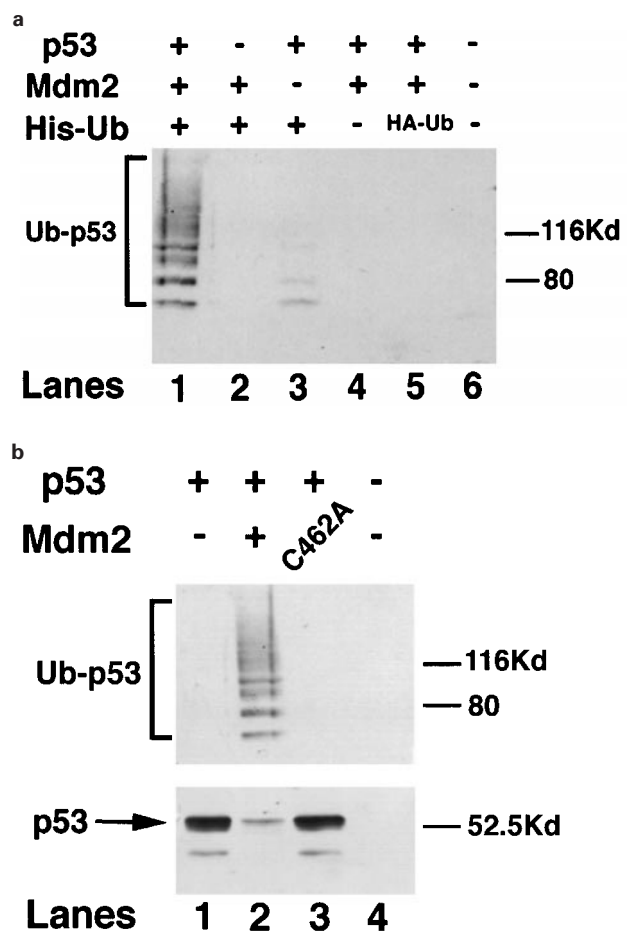


Figure 1 An *in vivo* assay for Mdm2-dependent ubiquitination of p53. (a) H1299 cells were transfected with 1 μ g of p53 expression plasmid and 2 μ g of Mdm2 expression plasmid with or without 2 μ g of plasmid expressing His-tagged Ubiquitin or HA-tagged ubiquitin. Ubiquitinated products were separated and analysed with the DO1 antibody against p53. (b) Cells were transfected with 1 μ g of p53 expression vector, 2 μ g of plasmid expressing His-tagged Ubiquitin expression plasmid and 2 μ g of Mdm2 expression plasmid or 2 μ g of Mdm2C462 expression plasmid. Ubiquitinated p53 was analysed as above. Total p53 levels are shown in the lower panel

Eluates were then separated by SDS-PAGE, transferred to a nitrocellulose filter and ubiquitinated p53 was detected by probing the membrane with the highly specific DO-1 anti-p53 antibody. Figure 1a (lane 1) shows a ladder of ubiquitinated p53 forms obtained using this procedure. This was not detected when p53 expression was omitted (lane 2) or when His₆-tagged ubiquitin expression was omitted (lane 4) or replaced with haemagglutinin-tagged ubiquitin expression (lane 5), demonstrating the specificity of the assay. A low level of ubiquitination of p53 was observed when Mdm2 overexpression was omitted (lane 3). This could be due to the endogenous hMdm2 present in the H1299 cell line or to an Mdm2-independent ubiquitinating activity. The cysteine residue at position 464 of the human Mdm2 has been shown to be essential for the *in vitro* ubiquitination and the degradation of p53 (Honda *et al.*, 1997; Fang *et al.*, 2000). Accordingly, the corresponding mutant form of the murine Mdm2 (Mdm2C462A) was not able to promote the ubiquitination and degradation of p53 in our *in vivo* assay (Figure 1b) even though this mutant is well expressed, as shown in Figure 3d.

Effect of p14ARF overexpression and the proteasome inhibitor MG132 on the levels of ubiquitinated p53 in vivo

It has been shown that p14ARF overexpression rescues p53 from degradation by Mdm2 (see Introduction). However, the exact mechanism by which p14ARF performs this function *in vivo* is not yet clear. Having established an *in vivo* p53 ubiquitination assay, we investigated the effect of p14ARF overexpression on Mdm2-mediated ubiquitination and degradation of p53 *in vivo* and compared it to the effects of the proteasome inhibitor MG132. H1299 cells were transfected with constant amounts of constructs coding for wild-type human p53, murine Mdm2, His₆-ubiquitin and increasing amounts of p14ARF. Thirty-six hours post-transfection cells were harvested. An aliquot was lysed in NP40 buffer and used for direct Western blot analysis in order to monitor the total amount of p53 in the cells (see Materials and methods). The remainder of the cells was used for the His₆-ubiquitin-Ni²⁺-agarose purification assay. In this way we could compare the effects of p14ARF overexpression and MG132 on the levels of ubiquitinated p53 to their effects on the levels of total p53.

Co-transfection of p53 and Mdm2 resulted in an increase in the amount of ubiquitinated p53 (Figure 2a, lane 2) and the reduction of the total p53 protein levels (Figure 2a, compare lanes 1 and 2). Treatment of cells with the proteasome inhibitor MG132 for 3 h protected p53 from Mdm2-mediated degradation (Figure 2a, compare lanes 2 and 3). This increase in p53 protein levels was accompanied by an accumulation of ubiquitinated p53 without changing the ubiquitination profile (Figure 2a, compare lanes 2 and 3).

To obtain a more quantitative picture of the ratio of ubiquitinated to total p53 protein we developed a

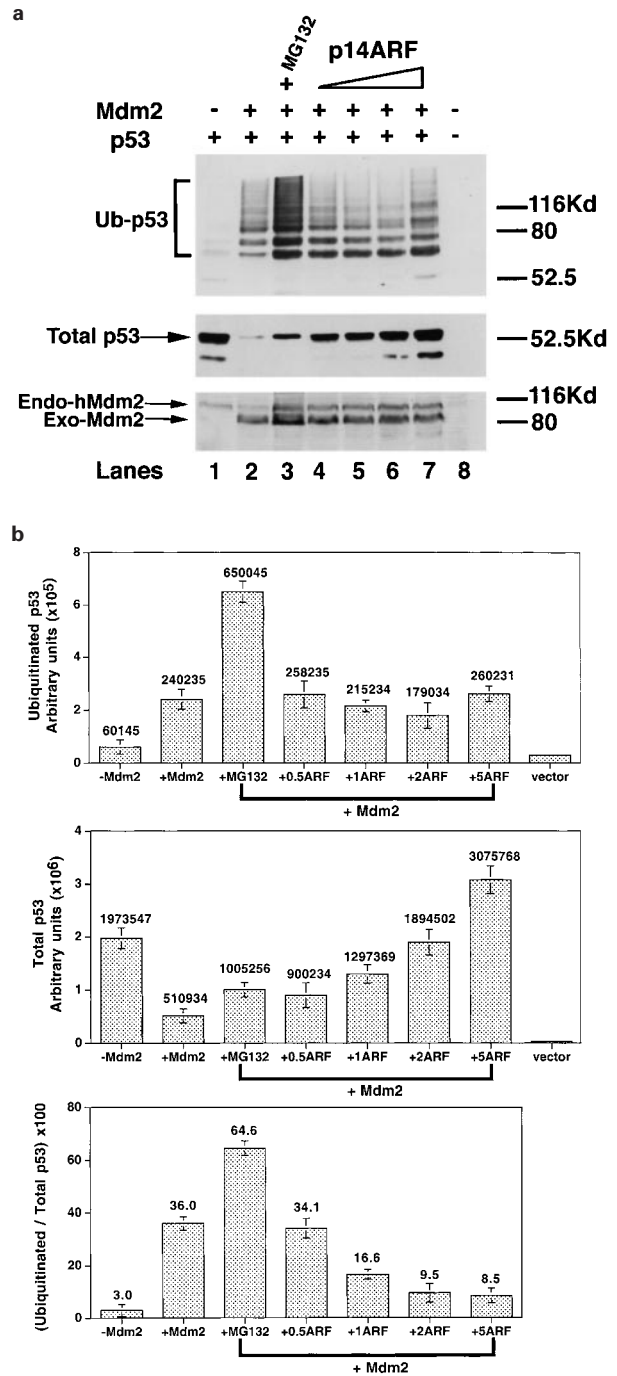


Figure 2 p14ARF overexpression decreases the proportion of ubiquitinated p53. (a) H1299 cells were transfected with 1 μ g of p53, 2 μ g of plasmid expressing His-tagged Ubiquitin and 2 μ g of Mdm2 expression plasmid. In lane 3, cells were treated with 30 μ M MG132 for 3 h. In lanes 4 to 7, increasing amounts of the p14ARF expression plasmid (0.5, 1, 2 or 5 μ g) were also transfected. Ubiquitinated p53 was analysed in the upper panel as described above. Total p53 and Mdm2 were analysed in the lower panels by direct Western blot. (b) Quantification of the proportion of ubiquitinated p53 in the experiment presented in panel a by the ELISA based method described in Materials and methods

sensitive two-site ELISA. Ninety-six-well plates were coated with DO1 anti-p53 monoclonal antibody to capture p53 protein from total cell extracts or ubiquitinated eluates. DO1-associated p53 protein was then detected by anti-p53 rabbit polyclonal serum CM-1 (see Materials and methods). As shown in Figure 2b, co-expression of Mdm2 resulted in an increase in the ratio of ubiquitin conjugated p53 to total p53. As would be expected, blocking proteasomal activity not only increased the levels of ubiquitinated p53 further but also resulted in a dramatic rise in the ratio of ubiquitinated p53 to total p53.

Increasing amounts of transfected p14ARF expression plasmid blocked Mdm2-mediated degradation of p53 and restored p53 levels (Figure 2a, compare lane 2 with lanes 4–7). This was associated with changes in the ubiquitination profile of p53. Increasing amounts of p14ARF preferentially decreased the appearance of slow migrating, ubiquitinated forms of p53 relative to the faster migrating forms (Figure 2a, compare lane 2 with lanes 4–7). The electrophoretic mobility of the faster migrating conjugates suggests that they probably correspond to mono- and di-ubiquitinated forms of p53. At the highest dose of p14ARF expression plasmid (Figure 2a, lane 7), the levels of total p53 increased further and the pattern of higher molecular weight ubiquitin-p53 conjugates was different from the pattern obtained in the absence of p14ARF. At this high dose of p14ARF expression, novel slow migrating forms appeared. Whether the slow mobility forms correspond to p53 modified by polyubiquitin or to p53 modified by monoubiquitin at multiple sites (Rodriguez *et al.*, 2000) needs to be studied.

In all experiments, p14ARF overexpression decreased the ratio of ubiquitinated to total p53 (Figure 2b). This decrease in the ratio of ubiquitinated p53 to total p53 occurred even at the higher dose of p14ARF expression despite the slight increase in the absolute levels of ubiquitinated p53 at this dose (Figure 2b, upper panel) and the appearance of a novel pattern of p53 ubiquitination described above (Figure 2a). Therefore, from this set of experiments we conclude that p14ARF overexpression attenuates Mdm2-mediated ubiquitination of p53 *in vivo* and preferentially decreases the levels of polyubiquitinated p53.

In order to gain information about the mechanisms by which p14ARF decreases the Mdm2-mediated ubiquitination and degradation of p53 we analysed their effect on the levels of Mdm2 protein. Full-length Mdm2 levels in the same total cell extracts from the experiment described above were analysed by Western blot. MG132 increased the levels of exogenous Mdm2 as well as of endogenous hMdm2 (Figure 2a, lower panel, compare lanes 2 and 3) indicating that Mdm2 is targeted for degradation by the proteasome in this cell system. Exogenous Mdm2 levels were only slightly increased by overexpression of p14ARF (Figure 2a, compare lane 2 with lanes 4–7). Instead, as discussed below and previously shown by Stott *et al.* (1998), the

levels of exogenous human Mdm2 were highly increased by the overexpression of p14ARF (Figure 3a). The reason for this difference between the response of the murine and the human Mdm2s to p14ARF overexpression is unknown. Endogenous hMdm2 levels were also increased when p14ARF was overexpressed (Figure 2a, compare lane 2 with lanes 4–7). This increase in the levels of endogenous hMdm2 could be due to the activation of p53-dependent transcription induced by p14ARF.

When setting up this system we used the mouse Mdm2 because the construct expressing the murine protein is more potent than the human Mdm2 (hMdm2) expression vector in promoting the degradation of p53. However, the human form also induced the ubiquitination and degradation of p53 in our assay (Figure 5b, compare lanes 1 and 2) as reported by others using different cell systems (Fang *et al.*, 2000; Geyer *et al.*, 2000). Furthermore, p14ARF had the same effect as on the pattern of p53 ubiquitination when the human Mdm2 was used (not shown).

Analysis of the effects of p14ARF overexpression on hMdm2 levels

The effect of p14ARF on the stability of Mdm2 has been an area of controversy as described in the Introduction section. To exclude the possible effects of p53 expression on the levels of Mdm2 we analysed the effect of p14ARF overexpression on Mdm2 levels in a p53 negative background. In H1299 cells, which lack endogenous p53, we found that p14ARF overexpression, like MG132, very efficiently increases the levels of exogenous hMdm2 (Figure 3a, lower panel). The same effect of p14ARF overexpression on hMdm2 levels was also observed when the cells were lysed directly in SDS-PAGE loading buffer (not shown) excluding the possibility of p14ARF-induced changes of the solubility of hMdm2. This effect of p14ARF on the levels of murine Mdm2 is weaker also in the absence of p53 for unknown reasons as mentioned before (Figure 3d). Overexpressed p14ARF did not increase the levels of ectopically expressed green fluorescent protein (GFP) indicating that p14ARF overexpression does not affect the transcription or translation of ectopically expressed proteins significantly (Figure 3b, left panel). Conversely, the overexpression of GFP did not increase the levels of hMdm2 (Figure 3b, right panel) indicating that protein overexpression does not significantly affect the levels of hMdm2.

Therefore, we have established that p14ARF overexpression increases the levels of ectopically expressed hMdm2 protein and distinguished this effect from a possible effect on endogenous hMdm2 expression due to activation of the transcriptional activity of p53.

This ability of p14ARF overexpression to increase the levels of exogenous hMdm2 in the absence of wild-type p53 depended on the ability of p14ARF to bind to

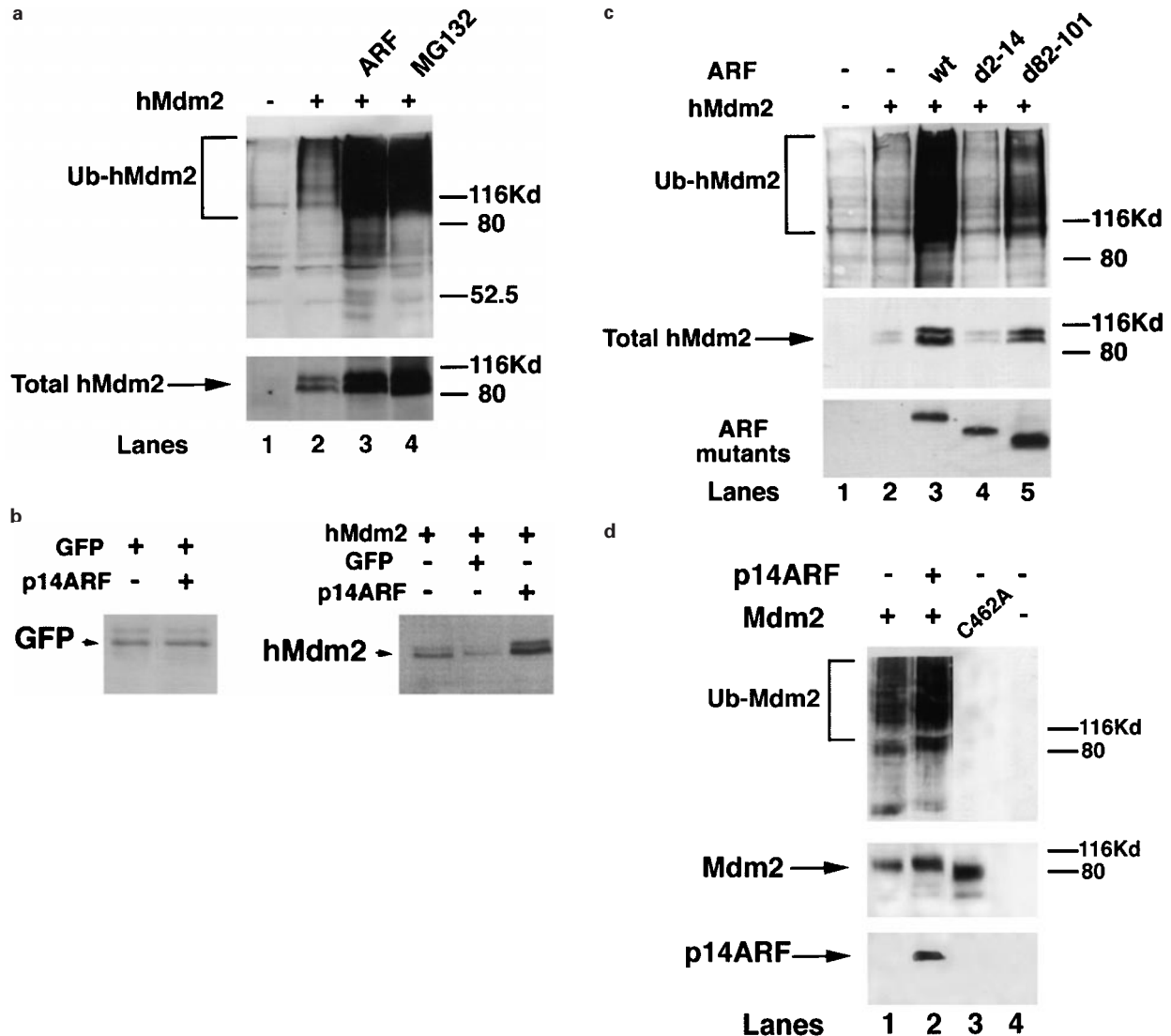


Figure 3 Effect of p14ARF overexpression on the levels and ubiquitination of hMdm2 in the absence of p53. (a) H1299 cells were transfected with 5 μ g of hMdm2 expression vector and with 2 μ g of plasmid expressing His-tagged Ubiquitin, together with 5 μ g of control pcDNA3 vector or p14ARF expression plasmid. Where indicated, cells were treated with 30 μ M MG132 for 3 h. Ubiquitinated products were separated and analysed with the 4B2 antibody against Mdm2. Total hMdm2 was analysed by direct Western blot in the lower panel. (b) H1299 cells were transfected with 5 μ g of hMdm2, GFP or p14ARF expression plasmids. hMdm2 and GFP were analysed by direct Western blot. (c) Effect of p14ARF mutants on ectopically expressed hMdm2. H1299 cells were transfected with 5 μ g of hMdm2 in the presence or absence of wild-type p14ARF or deletion mutant p14ARF (d2-14 and d82-101) expression plasmids. Ubiquitinated and total hMdm2 were analysed as above. p14ARF and p14ARF mutant levels are shown in the lower panel. (d) H1299 cells were transfected with 5 μ g of murine wild-type Mdm2 or Mdm2 C462A expression vectors and with 2 μ g of plasmid expressing His-tagged Ubiquitin, together with either 5 μ g of control pcDNA3 vector or p14ARF expression plasmid. Ubiquitinated and total Mdm2 were analysed as above.

hMdm2 as demonstrated by using p14ARF deletion mutants kindly provided by J Weber and C Sherr and described in (Weber *et al.*, 2000). The p14ARFd2-14 deletion mutant lacking the N-terminal region of p14ARF did not significantly increase hMdm2 levels (Figure 3c, bottom panel). This mutant has been previously reported to be deficient for hMDM2 binding and for protecting p53 from Mdm2-mediated degradation (Weber *et al.*, 2000).

p14ARF overexpression does not reduce the levels of ubiquitinated Mdm2 *in vivo*

Mdm2 has been reported to exert an auto-ubiquitination activity *in vitro* (Honda and Yasuda, 1999; Midgley *et al.*, 2000). To investigate the ubiquitination of Mdm2 *in vivo*, we followed the same approach as for the p53 ubiquitination assay. His₆-ubiquitinated eluates and total cell extracts were used for Western blot analysis

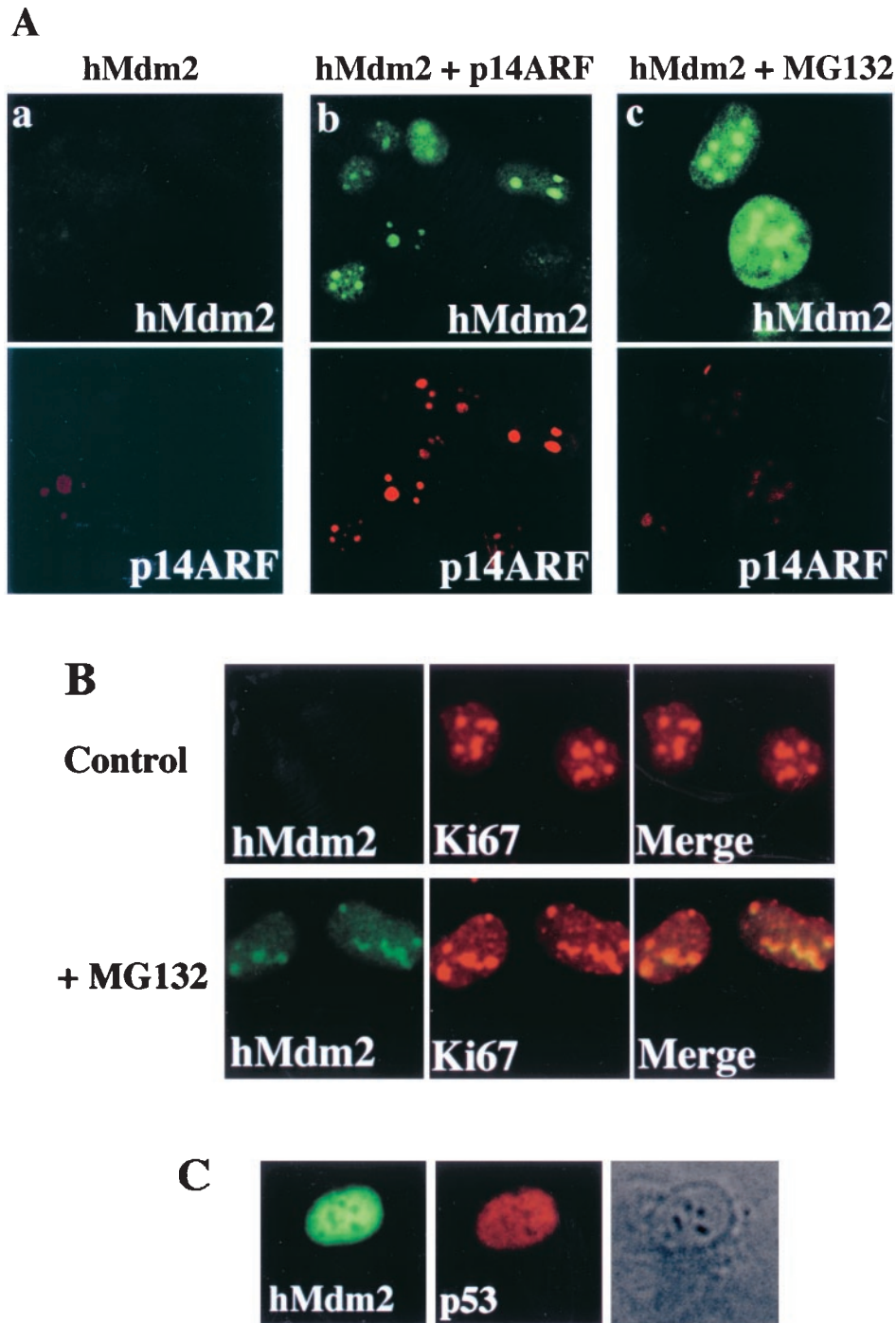


Figure 4 Proteasome inhibition induces the accumulation of hMdm2 in the nucleolus. (a) H1299 cells were transfected with a plasmid expressing hMdm2, together with pcDNA3 control vector (a), together with a vector expressing p14ARF (b) or treated with the proteasome inhibitor MG132 (30 μ M for 3 h) (c) and analysed by indirect immunofluorescence using the 4B2 antibody against hMdm2 and the rabbit polyclonal antibody against p14ARF. (b) U2OS cells, which are negative p14ARF expression, were left untreated or treated with MG132 (30 μ M for 3 h). Endogenous hMdm2 was detected by immunofluorescence as above. Nucleoli were detected staining with an antibody against the nucleolar antigen Ki67. To avoid artefacts derived from cross reactivity of the antibodies, control stainings using each antibody separately were also performed in all experiments (not shown). (c) P53 impairs the nucleolar localization of Mdm2 induced by p14ARF overexpression and by MG132. U2OS cells were transfected with wild-type p53 expression plasmid and treated with MG132. Mdm2 and p53 were detected with 4B2 mouse monoclonal antibody and the CM1 rabbit serum, respectively

using the 4B2 anti-(h)Mdm2 monoclonal antibody. Figure 3a (top panel) and d show that ubiquitinated human and murine Mdm2 can readily be detected with this assay. As a control (Figure 3d), we used the Mdm2 C462A mutant which lacks the ability to ubiquitinate p53 *in vitro* and *in vivo* (Figure 1b). As shown in Figure 3d, we did not detect significant levels of this mutant conjugated to ubiquitin. The same result was obtained using the corresponding human Mdm2 C464A mutant (not shown). The cysteine residue at position 462 of Mdm2 is thus necessary for the ubiquitination of Mdm2 in our system. This result also suggests that under these conditions Mdm2 is not significantly ubiquitinated by the low levels of endogenous hMdm2 in H1299 cells or by another ubiquitination activity. However, we cannot rule out that the cysteine residue at position 462 is necessary for Mdm2 to be recognized as a substrate for ubiquitination.

As reported previously and as described above, Mdm2 levels are sensitive to proteasome inhibitors both in the presence as well as in the absence of p53 (An *et al.*, 1998). Here we show that the addition of MG132 in the ubiquitination assay also increased the levels of ubiquitinated hMdm2 (Figure 3a, top panel). Although other forms of Mdm2 degradation cannot be ruled out, these observations confirm that hMdm2 is constitutively ubiquitinated and degraded by the proteasome in this cell system.

It has been previously suggested that p14ARF can decrease the autoubiquitination activity of Mdm2 *in vitro* (Honda and Yasuda, 1999; Midgley *et al.*, 2000). Therefore, one way in which p14ARF could increase the levels of Mdm2 protein is by inhibiting the ubiquitination of Mdm2. However, here we show that p14ARF overexpression does not significantly decrease the levels of hMdm2 ubiquitination *in vivo* under the conditions where (h)Mdm2-dependent ubiquitination of p53 was affected by overexpression of p14ARF. Co-expression of p14ARF clearly increased the levels of the hMdm2 protein (Figure 3a, bottom panel) but this was accompanied by an increase in the levels of ubiquitinated hMdm2 (Figure 3a, top panel). The same effect of p14ARF was obtained when p53 was also overexpressed in this assay (data not shown). A similar result was obtained using the murine Mdm2 (Figure 3d). In this case, p14ARF overexpression increased the levels of ubiquitinated Mdm2 to a higher extent than it increased the levels of total Mdm2. The analysis of the hMdm2 ubiquitination patterns obtained in the presence of overexpressed p14ARF deletion mutants (Figure 3c, top panel) revealed a good correlation between the ability of the different p14ARF forms to increase the levels of total hMdm2 and of ubiquitinated hMdm2 and indicates that the accumulation of the ubiquitinated forms of hMdm2 induced by p14ARF depends on efficiency of the binding of p14ARF to hMdm2. While the p14ARF d2-14 deletion mutant is inactive in increasing the levels of hMdm2 and the levels of ubiquitin-conjugated hMdm2, the p14ARF d82-101 deletion mutant has an

intermediate effect on hMdm2 levels and ubiquitination.

Proteasome inhibition induces the localization of hMdm2 in the nucleolus like p14ARF

Since the proteasome inhibitor MG132 and p14ARF overexpression had similar effects on the levels and ubiquitination of Mdm2 we compared the effect of these two agents on its subcellular localization. In agreement with previously reported data (Weber *et al.*, 1999) we observed that p14ARF overexpression causes the localization of exogenous human and mouse Mdm2 in the nucleolus of H1299 (Figure 4a) and U2OS cells (data not shown). In the absence of p53, MG132 increased the levels of hMdm2 in H1299 cells (see above) and also resulted in the appearance of hMdm2 in the nucleolus (Figure 4a). This localization was confirmed by immunostaining with two monoclonal antibodies against hMdm2 (4B2 and 3G5). The same result was obtained transfecting the murine Mdm2 expression plasmid (not shown). Since H1299 cells contain endogenous p14ARF (Figure 4a) we tested the effect of MG132 on the localization of hMdm2 in U2OS cells which have been reported to be negative for p14ARF expression (Stott *et al.*, 1998). As shown in Figure 4b, proteasome inhibition increased the levels of endogenous hMdm2 in the nucleolus of the U2OS cells. In a control experiment we confirmed that after the incubation with MG132, U2OS cells were negative for p14ARF expression (not shown). These results show that proteasome inhibition, like p14ARF permits to detect hMdm2 in the nucleolus.

The nucleolar localization of hMdm2 induced by p14ARF overexpression is known to be impaired by the presence of overexpressed p53 (Weber *et al.*, 1999). Using the system described in Figure 4b, we investigated whether the MG132-dependent nucleolar localization of hMdm2 was also decreased by the overexpression p53. As shown in Figure 4c, the presence of ectopically expressed p53 in U2OS cells impaired the detection of hMdm2 in the nucleolus. A similar effect was observed with H1299 cells transfected with plasmids expressing p53 and hMdm2 and treated with MG132 (data not shown).

The nucleolar localization signal in Mdm2 is not required for the ubiquitination of Mdm2

We have shown that both with p14ARF overexpression and proteasome inhibition there is a correlation between the appearance of nucleolar staining and the accumulation of ubiquitinated hMdm2. Therefore, we asked whether the nucleolar localization of hMdm2 is necessary for the ubiquitination of hMdm2. For this purpose we tested the effect of p14ARF on the levels and ubiquitination of a mutant of hMdm2 which is defective for nucleolar localization even in the presence of p14ARF as previously described by Lohrum *et al.* (2000a) and reproduced by us in the H1299 cell system (data not shown). As shown in Figure 5a, this mutant

(hMdm2 NoLS) was as effectively ubiquitinated as the wild-type hMdm2. This implies that the nucleolar localization of hMdm2 is not necessary for ubiquitination of hMdm2. This mutant was also stabilized by p14ARF (Figure 5a) as previously described (Lohrum *et al.*, 2000). This must mean that this mutant hMdm2 protein is subject to ubiquitin dependent degradation outside of the nucleolus and that p14ARF can inhibit this process without inducing the localization of hMdm2 to the nucleolus.

We then tested the effects of the hMdm2 NoLS mutant on the ubiquitination profile of p53. As reported previously (Lohrum *et al.*, 2000), this mutant was defective in promoting the degradation of p53 (Figure 5b). The levels of polyubiquitinated p53 and

the ratio of ubiquitinated to total p53 obtained with this mutant were lower than those obtained with wild-type hMdm2 (Figure 5b). Unlike the (h)Mdm2 C462A mutant, the hMdm2 NoLS mutant was defective in promoting the appearance of polyubiquitinated forms of p53 but was not greatly impaired in its ability to mediate monoubiquitination of p53. Whether this defect in increasing the levels of polyubiquitinated p53 is related to the inability of this hMdm2 mutant to reach the nucleolus or whether it is due to a possible defect of this mutant to catalyse the polyubiquitination of p53 needs to be established by *in vitro* assays. Despite this limitation, this experiment supports the notion that the monoubiquitination of p53 does not require the region containing the nucleolar localization signal of hMdm2 (residues 466 to 473) and that the monoubiquitinated and polyubiquitinated forms of p53 differ in their sensitivity to p14ARF overexpression (see Figure 2a) and to the lack of the nucleolar localization region in Mdm2.

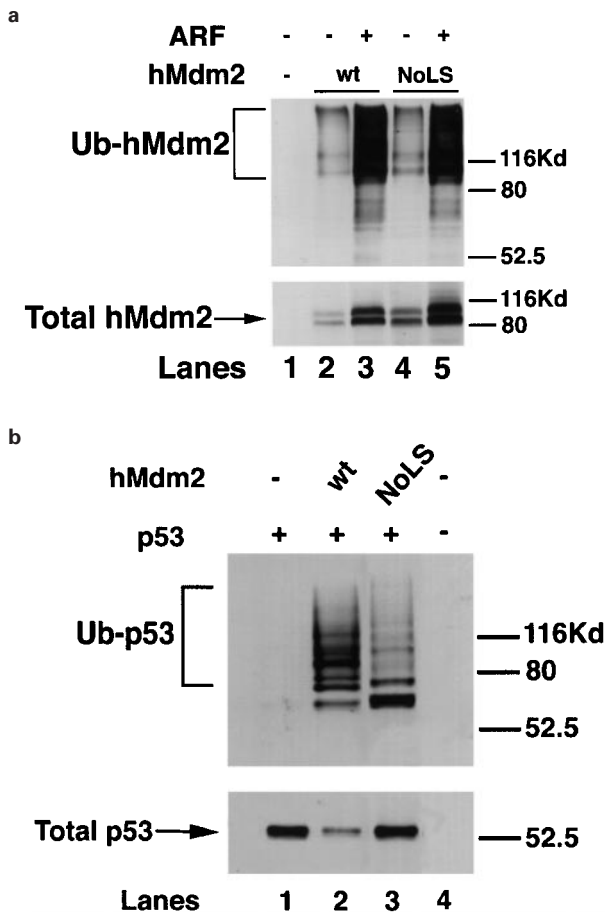


Figure 5 (a) Ubiquitination of the hMdm2 mutant defective for nucleolar localization hMdm2NoLS. H1299 cells were transfected with 2 μ g of plasmid expressing His-tagged Ubiquitin and 5 μ g of hMdm2 or hMdm2NoLS expression plasmid. In lanes 3 and 5, 5 μ g of p14ARF expression plasmid were also transfected. Ubiquitinated hMdm2 was analysed by direct Western blot in the upper panel. Total hMdm2 was analysed by direct Western blot in the lower panel. (b) Effect of hMdm2NoLS on the levels of p53 ubiquitination. H1299 cells were transfected with 1 μ g of p53, 2 μ g of plasmid expressing His-tagged Ubiquitin and 5 μ g of hMdm2 or hMdm2NoLS expression plasmid. Ubiquitinated p53 was analysed in the upper panel. Total p53 was analysed by direct Western blot in the lower panels

Discussion

In this study we have compared the effect of p14ARF overexpression on the levels and ubiquitination of p53 and Mdm2 *in vivo*. For this purpose we have developed an *in vivo* ubiquitination assay for p53 and Mdm2. With this assay we have demonstrated differences in the effect of p14ARF overexpression on the levels of ubiquitinated p53 and on the levels of ubiquitinated Mdm2.

The effect of p14ARF on Mdm2-mediated degradation of p53 was associated with changes in the pattern of Mdm2-dependent ubiquitination of p53. p14ARF had a general inhibitory effect on Mdm2-dependent ubiquitination of p53 but preferentially decreased the levels of polyubiquitinated p53. It has been shown that polyubiquitinated rather than the mono- or di-ubiquitinated proteins are efficiently recognized and degraded by the proteasome (Thrower *et al.*, 1999). Thus, decreasing the levels of polyubiquitinated p53 would provide a mechanism by which p14ARF could prevent Mdm2-dependent targeting of p53 for proteasomal degradation. The effect of p14ARF on p53 ubiquitination was clearly distinct from that of MG132 which caused accumulation of ubiquitinated p53 because it inhibits the degradation of ubiquitinated p53 by the proteasome rather than blocking ubiquitination.

p14ARF has been shown to inhibit the Mdm2-mediated ubiquitination of p53 *in vitro* (Honda and Yasuda, 1999; Midgley *et al.*, 2000). Therefore, inhibition of the E3 ubiquitin ligase activity of Mdm2 by p14ARF could play a major role in the protection of p53 from Mdm2-mediated degradation. However, the reported data do not exclude other models to explain the effect of Mdm2-dependent degradation of p53. p14ARF could impair the ubiquitination activity of Mdm2 on p53 by promoting the sequestration of Mdm2 in the nucleolus away from p53 in line with the

model proposed by Weber *et al.* (1999). p14ARF could also inhibit the ubiquitination of p53 by Mdm2 by blocking the export of p53/Mdm2 complexes from the nucleus to the cytoplasm in agreement with the models proposed by Zhang and Xiong (1999) and Tao and Levine (1999). Additionally, we think that it is important to notice that our assay does not allow one to distinguish between the effect of p14ARF on the E3 ligase activity of Mdm2 on p53 from the possible effect of p14ARF on the deubiquitination of p53. Therefore, we cannot exclude that p14ARF could be promoting deubiquitination of polyubiquitinated p53 or inhibiting the access of polyubiquitinated p53 to the proteasome allowing deubiquitinating enzymes to act on polyubiquitinated p53. This could explain the preferential decrease in the polyubiquitinated forms of p53 in the presence of p14ARF.

In agreement with a previous report (An *et al.*, 1998), we observed that MG132 increased the level of Mdm2 independently of the presence of p53. We also showed that this accumulation is accompanied by an increase in the levels of ubiquitinated Mdm2. This indicates that Mdm2 is targeted by ubiquitination to the proteasome. Furthermore, the ubiquitination of Mdm2 was abolished by mutation of the cysteine residue at position 462 of murine MDM2. The same effect was observed by mutating the equivalent residue (464) in human Mdm2 (D Xirodimas, data not shown) in agreement with previous *in vitro* data (Fang *et al.*, 2000). The 464 cysteine residue is in a region of Mdm2 that is conserved in other ubiquitin E3 ligases and is necessary for the ubiquitination of p53 by Mdm2 (Honda *et al.*, 1997; Honda and Yasuda, 2000; Fang *et al.*, 2000). This suggests that Mdm2 ubiquitination *in vivo* is due to its own E3 ubiquitin ligase activity.

We then examined the effect of p14ARF overexpression on the levels of total Mdm2 protein and on the levels of ubiquitinated Mdm2. p14ARF overexpression caused an increase in the levels of ectopically expressed Mdm2 independently of the presence of p53. This p14ARF-dependent increase in Mdm2 levels was not associated with a decrease in the overall level of Mdm2 ubiquitination or with changes in the pattern of Mdm2 ubiquitinated species. The effect of p14ARF overexpression on Mdm2 levels and ubiquitination was very similar to the effect of proteasome inhibition by MG132. These data suggest that p14ARF may exert its effects on Mdm2 by inhibiting degradation of Mdm2 by the proteasome rather than through effects on the overall ubiquitination of Mdm2. However, we cannot exclude the possibility that p14ARF could cause subtle changes in the ubiquitination of Mdm2 that are not detected in our assay. It is possible that ubiquitination of a specific site is sensitive to inhibition by p14ARF and that this modification is required for the effective degradation of Mdm2. Alternatively, p14ARF could be protecting Mdm2 from forms of degradation other than one involving the proteasome, for example caspase cleavage (Pochampally *et al.*, 1998).

Although the mechanism by which p14ARF overexpression induces the accumulation of Mdm2 remains to be elucidated, our results are in contrast with *in vitro* experiments which have led to the proposal that p14ARF inhibits the overall ubiquitination of Mdm2 (Honda and Yasuda, 1999; Midgley *et al.*, 2000). The factors involved in the *in vivo* ubiquitination of Mdm2 have not been unequivocally established and it is not clear how well the conditions used in the *in vitro* assays recapitulate the *in vivo* situation. The difference between the *in vitro* and *in vivo* results could also be explained if either the ubiquitination of Mdm2 *in vivo* occurs before its interaction with p14ARF or if the ubiquitination of Mdm2 *in vivo* is due to a ubiquitination activity that is not inhibited by p14ARF. Since the E3 ligase inactive Mdm2 C462 mutant is not effectively ubiquitinated in our system, a putative p14ARF-insensitive ubiquitination of Mdm2 is at first sight unlikely. However, it is possible that the ubiquitination of Mdm2 by an activity that is not sensitive to p14ARF requires an intact cysteine residue at position 462 in the murine Mdm2 (464 in the human version).

Since p14ARF and MG132 have very similar effects on the levels and ubiquitination of Mdm2 we compared their effects on the subcellular localization of Mdm2. p14ARF has been proposed to sequester Mdm2 in the nucleolus (Weber *et al.*, 1999). We found that MG132, like p14ARF, also increased the appearance of Mdm2 in the nucleolar compartment. Mdm2's ability to reach the nucleolus in the presence of MG132 did not depend on p14ARF since proteasome inhibition induces the localization of Mdm2 in the nucleolus in the absence of p14ARF expression. Therefore, we propose that, at least in part, p14ARF overexpression could result in the appearance of Mdm2 in the nucleolus by simply increasing the levels of the protein or of its ubiquitinated forms. However, as reported by Weber *et al.* (2000), p14ARF could additionally induce a conformational change in the structure of Mdm2 that potentiates the exposure of the nucleolar localization signal in Mdm2 described by Lohrum *et al.* (2000a). The nucleolar localization of Mdm2 induced by p14ARF overexpression was shown to be inhibited by the overexpression of p53 (Weber *et al.*, 1999). Similarly, overexpression of p53 also inhibited the nucleolar localization of endogenous Mdm2 induced by MG132.

The similarity between the effect of p14ARF overexpression and MG132 on the levels, ubiquitination and localization of Mdm2 could suggest that both agents inhibit the degradation of Mdm2 by a mechanism that involves the nucleolar compartment. However, an hMdm2 mutant defective for nucleolar localization was also stabilized by p14ARF overexpression as previously reported by Lohrum *et al.* (2000). This does not exclude the possibility that degradation can involve a nucleolar step but supports the suggestion that the nucleolar localization of hMdm2 is not essential for its destabilization. Furthermore, this observation also suggests that

p14ARF does not protect Mdm2 from degradation by sequestering it in the nucleolar compartment.

Since p14ARF and MG132 both increased the levels of ubiquitinated Mdm2 and both induced the localization of Mdm2 in the nucleolus we questioned whether the nucleolar localization was necessary to detect Mdm2 ubiquitination. This was shown not to be the case since a hMdm2 mutant defective for its nucleolar localization (Lohrum *et al.*, 2000) was as effectively ubiquitinated in our system as wild-type hMdm2. We also showed that, like the overexpression of p14ARF, the mutation of the nucleolar localization signal of hMdm2 preferentially impaired the ability of Mdm2 to increase the levels of polyubiquitinated p53 over the monoubiquitinated forms of p53. Whether this mutation alters the capacity of Mdm2 to promote polyubiquitination *in vitro* needs to be established in order to confirm whether the ability of Mdm2 to reach the nucleolus is necessary for the effective ubiquitination and degradation of p53.

In summary, we have presented data showing that: (1) p14ARF overexpression stabilizes p53 by decreasing the levels of p53 ubiquitination. Differences in the sensitivity of the polyubiquitinated and monoubiquitinated forms of p53 to the presence of p14ARF were also detected; (2) p14ARF also increases the levels of Mdm2 but this is not associated with a decrease in the overall levels of ubiquitinated protein; (3) The accumulation of hMdm2 or its ubiquitinated forms by the inhibition of proteasome activity is sufficient to detect Mdm2 in the nucleolus; (4) The nucleolar localization of Mdm2 is not necessary for the ubiquitination or degradation of Mdm2 or the conjugation of p53 to monoubiquitin. However, the region containing the nucleolar localization signal of hMdm2 is required for the effective polyubiquitination of p53.

These studies emphasize key differences in the regulation of Mdm2 via its ubiquitination from the regulation of p53 via Mdm2-dependent ubiquitination. The capacity to differentially regulate these two events may allow an even more subtle control on p53 function to be established.

Materials and methods

Cells, antibodies and reagents

H1299 and U2OS cells were obtained from the ATCC and cultured in RPMI or DMEM medium, respectively, supplemented with 10% FCS and gentamycin at 37°C, 5% CO₂ in a humidified atmosphere.

P53 was detected using the DO1 mouse monoclonal antibody (Stephen *et al.*, 1995) or the CM-1 anti-p53 rabbit serum (Midgley *et al.*, 1992), Human and mouse Mdm2 were detected using the 4B2 mouse monoclonal antibody (Chen *et al.*, 1993). Rabbit anti-p14ARF serum (IPI) was a kind gift from Dr K Vousden (Stott *et al.*, 1998). Ki67 was detected using the rabbit polyclonal serum from DAKO. MG132 was purchased from Calbiochem.

Plasmids

Expression from all constructs was under the control of CMV promoter. The pCOC-X2 Mdm2, the hMdm2, p14ARF and the His₆-ubiquitin expressing constructs were a kind gift from Dr M Oren, Dr A Levine, Dr K Vousden and Dr S Mittnacht, respectively. The vector expressing the Mdm2 C464A mutant was derived from the pCOC-X2 Mdm2 plasmid by site directed mutagenesis. P53 cDNA was cloned into the *EcoRI* and *XhoI* sites of pCDNA3.

Transfection of cells and purification of His-tagged ubiquitin conjugates

H1299 cells were cultured in RPMI supplemented with 10% foetal calf serum (FCS) and gentamycin. 1 × 10⁶ of H1299 cells were seeded on 10 cm tissue culture plates and transfected using the calcium-phosphate method. At least 1 h before transfection, the cells were cultured in DMEM 10% FCS medium. Eighteen hours after transfection, the medium was changed back to RPMI 10% FCS. Purification of His₆-ubiquitinated conjugates was performed as described in Rodriguez *et al.* (1999). Thirty-six hours post transfection cells were washed twice with PBS and scraped in 1 ml of PBS. Twenty per cent of cell suspension was used for direct Western blot analysis (see below). The remainder was lysed in 6 ml of 6 M guanidinium-HCl, 0.1 M Na₂HPO₄/NaH₂PO₄, 0.01 M Tris/HCl, pH 8.0, 5 mM imidazole and 10 mM β-mercaptoethanol. Seventy-five μl of Ni²⁺-NTA-agarose beads (Qiagen) were then added and lysates were rotated at room temperature (RT) for 4 h. The beads were successively washed for 5 min in each step at room temperature with 750 μl of each of the following buffers: 6 M guanidinium-HCl, 0.1 M Na₂HPO₄/NaH₂PO₄, 0.01 M Tris/HCl, pH 8.0 plus 10 mM β-mercaptoethanol; 8 M urea, 0.1 M Na₂HPO₄/NaH₂PO₄, 0.01 M Tris/HCl, pH 8.0, 10 mM β-mercaptoethanol; 8 M urea, 0.1 M Na₂HPO₄/NaH₂PO₄, 0.01 M Tris/HCl, pH 6.3, 10 mM β-mercaptoethanol (buffer A) plus 0.2% Triton X-100; buffer A and then buffer A plus 0.1% Triton X-100. After the last wash His₆-tagged ubiquitinated products were eluted by incubating the beads in 75 μl of 200 mM imidazole, 0.15 M Tris/HCl pH 6.7, 30% glycerol, 0.72 M β-mercaptoethanol, 5% SDS for 20 min at RT. The eluates were mixed in 1:1 ratio with 2 × Laemmli-buffer and analysed by Western blot analysis.

Lysis of cells

For direct Western blot analysis, the cell pellet obtained from the 20% cell suspension described before was lysed in NP-40 lysis buffer (150 mM NaCl, 50 mM Tris/HCl pH 8.0, 5 mM EDTA pH 8.0, 1% NP-40, 2 mM dithiothreitol (DTT), 2 mM phenylmethylsulfonyl fluoride (PMSF). After incubation on ice for 30 min, lysates were centrifuged at 14 000 r.p.m. and pellets were discarded. Protein concentration in the supernatant was determined by Bradford assay, and equal amounts of total protein was used for Western blot analysis. This procedure allows the detection of total p53 or Mdm2 levels. No significant amounts of p53 or Mdm2 were detected in the pellet fractions. No ubiquitinated forms were detected probably due to the action of deubiquitinating enzymes in the NP40 lysis buffer.

Western blot analysis

Samples were separated on 10% polyacrylamide-SDS gels. The gel was transferred overnight to nitrocellulose membrane and blocked in PBS-Tween-20 (0.1% v/v) with dry skimmed milk (5% w/v) (PBSTM) for 1 h at RT. The membrane was washed three times for 15 min with PBST before incubation with monoclonal mouse antibodies, (1 µg/ml in PBSTM) for 1 h at RT. The filter was washed as before and incubated with horseradish peroxidase (HRP) conjugated anti-mouse IgG (DAKO P161) at a dilution of 1:1000 in PBSTM for 1 h at RT. The membrane was washed and developed by ECL (Amersham).

Two-site ELISA

96-well flat bottomed tissue culture plates (Falcon 3296) were coated with 50 µl of purified DO1 anti-p53 monoclonal antibody (5 µg/ml). The wells were blocked for 1 h with PBSTM. Whole cell extracts in NP-40 lysis buffer or His₆-ubiquitinated conjugates eluted in 200 mM imidazole, 0.15 M Tris/HCl pH 6.7, 30% glycerol, 10 mM β-mercaptoethanol were diluted in PBSTM and added to the antibody coated wells for 2 h. Detection of p53 was performed by adding the polyclonal rabbit anti-p53 serum CM-1, 1:1000 in PBSTM for 1 h, followed by 1 h incubation with swine anti-rabbit horse radish peroxidase conjugated IgG (DAKO P217), 1:1000. Detection of the signal was performed by addition of Super Signal ELISA Pico Chemiluminescent substrate (Pierce) and the relative light units were measured with a Berthold microplate

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- luminometer LB 96V (~425 nm). All steps were carried out at RT and the plates were washed 5× with PBST between each incubation step. For each condition duplicates were performed and each experiment was repeated three times. Standard deviation values were derived from arithmetic means.

Immunofluorescence

H1299 or U2OS cells (1 × 10⁵/well) were seeded on two well NUNC permanox slides. Thirty-six hours after transfection using the calcium-phosphate method, cells were fixed with ice-cold methanol-acetone and incubated with primary antibodies followed by FITC conjugated Donkey anti-mouse or Texas Red conjugated Donkey anti-rabbit secondary antibodies (Jackson ImmunoResearch) as described in Lain et al., 1999.

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