Oligomerization of the Human ARF Tumor Suppressor and Its Response to Oxidative Stress*

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The tumor suppressor ARF plays an important role as an inhibitor of the Mdm2-mediated degradation of p53. Here we demonstrate that human ARF (p14ARF) can form homo-oligomers. The stability of the oligomers is favored by oxidizing agents in a reversible fashion and involves all three cysteine residues in p14ARF. Furthermore, the effect of p14ARF in clonogenic assays is moderately but reproducibly increased by the mutation of its cysteine residues. We also observed that altering the amino terminus of p14ARF resulted in the appearance of remarkably stable oligomers. This indicates that the amino terminus of p14ARF interferes with the ability of the protein to form multimeric complexes. These observations suggest that p14ARF activity may be linked to its oligomerization status and sensitive to the redox status of the cell.

In normal non-stressed cells p53 has a very short half-life (5–20 min) due to an autoregulatory feedback loop mechanism in which the Mdm2 protein plays a key role (reviewed in Refs. 1 and 2). It has been well established that wild type p53 acts as a transcriptional activator of the Mdm2 gene. In turn, Mdm2, which itself has a brief half-life due to its auto-ubiquitination activity, has the ability to interact with p53 and to function as a ubiquitin E3 ligase that promotes the conjugation of p53 to ubiquitin (3–5). This conjugation to ubiquitin serves as a tag that effectively targets p53 for degradation by the proteasome. In this way, in normal non-stressed cells, p53 is maintained at low levels, and cells are allowed to proliferate.

The ARF tumor suppressor (p14ARF in human, p19ARF in mouse) is encoded by the INK4/ARF locus (6). This small protein has been shown to inhibit degradation of p53 mediated by Mdm2 (7, 8). Several models have been proposed to explain this effect of p14ARF. In vitro biochemical studies indicate that p14ARF inhibits the ubiquitin E3 ligase activity of Mdm2 (9), (10) and a decrease in the levels of polyubiquitinated p53 by p14ARF was also shown in vivo (11). In addition, p14ARF was reported to sequester the p53-Mdm2 complex in discrete subnuclear compartments and to inhibit the nuclear export of the complex, a step suggested to be essential for p53 degradation (12). According to Tao and Levine (13) this export step could occur via the nucleolus. Finally, in another study, overexpressed p14ARF was shown to induce the localization of Mdm2 in the nucleolus, which is where ARF is primarily detected (14, 15) and proposed to sequester Mdm2 in this compartment. This would prevent its effects on p53 (15). Whether this mechanism is entirely responsible for the activation of p53 by p14ARF has been questioned by others (16–18). p53-independent tumor suppressor effects of ARF have also been described (19, 20).

ARF expression has been shown to be regulated at the transcriptional level (reviewed in Ref. 21). The expression of the ARF gene is induced by several oncogenic signals such as Myc, E1A, E2F, mutated ras, and v-Ab1. ARF expression is inhibited by Twist and Tbx-2 and is actively repressed during development by the Bmi repressor. Additionally, the human ARF promoter can be silenced by methylation or expression of wild type p53 (22). However, to our knowledge, there are no reports on the regulation of p14ARF function at the protein level.

Here we propose that p14ARF forms homo-oligomers and that these are stabilized by oxidative agents. Interest-ingly, the amino terminus of p14ARF, which is known to be critical for its function, can modulate the appearance of the oligomeric forms.

MATERIALS AND METHODS

Cells, Antibodies, and Reagents—H1299 and U2OS cells were obtained from the ATCC cultured in RPMI or Dulbecco’s modified Eagle’s medium, respectively, supplemented with 10% fetal calf serum and gentamicin at 37 °C, 5% CO2 in a humidified atmosphere. 4B2 and 2A10 are mouse monoclonal antibodies against Mdm2 (23). Human p53 was detected using the mouse monoclonal antibody DO1 (24). Rabbit anti-p14ARF serum (IPI) was a kind gift from Dr. K. Vousden (20). The rabbit serum against fibrillarin was a kind gift from Dr. A. Lamond. The mouse monoclonal antibodies against B23 (nucleophosmin) and the His tag were obtained from Zymed Laboratories Inc. and Novagen, respectively. Proteasome inhibitor MG132 was obtained from Calbiochem, and stock solutions were prepared in Me2SO.

Plasmids—Expression from all constructs was under the control of CMV promoter. pcMVMdM2 and pcDNA3p14ARF were a kind gift from Dr. A. Levine and Dr. K. Vousden, respectively. pcDNA3 β-galactosidase was a kind gift from Dr. R. Stad. All pcDNA3p14ARF derivatives were generated by standard site-directed mutagenesis. The vectors below.

Transfection of Cells—H1299 or U2OS cells were seeded on 10-cm tissue culture plates at a density of 9 × 104 cells per well and transfected using the calcium-phosphate method essentially as described previously (11). In all experiments, 1 μg of plasmid encoding the β-galactosidase protein was used as a transfection efficiency control. Equivalent amounts of CMV promoter in the transfections were maintained with the pcDNAs control vector. The amount of plasmid used in each transfection was topped up to 20 μg with the bacterial plasmid BlueScript. After 36 h cells were harvested in PBS, Cell pellets were washed with PBS and lysed using Nonidet P-40 lysis buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 1% (v/v) Nonidet P-40, and protease

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The abbreviations used are: E3, ubiquitin-protein isopeptide ligase; CMV, cytomegalovirus; PBS, phosphate-buffered saline; DTT, dithiothreitol; MOPS, 4-morpholinepropanesulfonic acid; IVT, in vitro transcription translation; RIP, radioimmune precipitation assay buffer.
inhibitors (Complete, Roche Molecular Biochemicals) for 15 min after which they were centrifuged for 20 min at 16,000 rpm, and the cell pellet was discarded. The volumes of the samples were adjusted to equivalent protein concentration and Laemmli buffer or Noveox loading buffer (with or without 0.1 M DTT) was added. Where indicated, cell pellets were directly lysed in Laemmli buffer or Noveox loading buffer supplemented or not with 0.1 M DTT. The quality of the transfections, sample preparations, and Western blot analysis were always monitored by the expression of β-galactosidase.

Western Blot Analysis—Samples were separated by denaturing electrophoresis on 4−12% (to detect p53 and Mdm2) or 12% (to detect p14ARF and all other antigens) Novex polyacrylamide gels run with MOPS buffer or with non-reducing agents. Similar results were obtained with Tris-glycine gels. Blots were transferred to nitrocellulose or Immobilon membranes and developed with the relevant primary antibodies and horseradish peroxidase-conjugated secondary antibodies as described in (11). Horseradish peroxidase activity was detected by ECL (Amersham Biosciences).

Immunoprecipitations—H1299 cells were transfected with 10 μg of pcDNA3p14ARF or pcDNA3p14ARFC15,100,123A together with 2.4 μg of recombinant hMdm2 obtained as described previously (10) and incubated for 30 min at 4°C. This mixture was divided into four aliquots, and the indicated antibodies, previously coupled to protein G (4B2 and DO2) or protein A (2A10 and 421) beads, were added. After 1 h of rotation at 4°C antibody-bound complexes were purified by centrifugation and extensive washing in Nonidet P-40 buffer. Samples were run on 12% Novex gels with MOPS buffer in non-reducing conditions. 35S-labeled bands were analyzed by autoradiography.

Preparation of Nucleoli—The protocol used was adapted from the one described previously (25). 3 × 10^6 H1299 cells were seeded. After 36 h, cells were harvested in PBS and cell pellets were resuspended in 3 ml of 10 mM Tris-HCl, pH 7.4, 10 mM NaCl, and 1.5 mM MgCl2. Cells were broken with the loose pestle of a Dounce homogenizer, and nuclei were sedimented by centrifugation at 228 × g for 5 min. Supernatants ("cytoplasmic" fraction) were concentrated with Centricon tubes and stored at −80°C. One-tenth volume (to detect p14ARF) or one-twentieth volume (to detect all other antigens) Novex polyacrylamide gels run with MOPS buffer with or without antioxidant agent. Similar results were obtained using classic Tris-glycine gels. Proteins were transferred to MOPS buffer with or without 0.1M DTT. The quality of the transfections, sample preparations, and Western blot analysis were always monitored by the expression of β-galactosidase.

p53 Reporter Assays—U2OS cells (1 × 10^5/well) were seeded on two well Nunc Permanox slides. 36 h after transfection using the calcium phosphate method, cells were fixed with ice-cold methanol-acetone and incubated with primary antibodies followed by fluorescein isothiocyanate-conjugated donkey anti-mouse or Texas Red-conjugated donkey anti-rabbit secondary antibodies (Jackson ImmunoResearch) as described previously (26).

RESULTS

Quantification of Recombinant p14ARF—The E. coli strain BLR(DE3) was transformed with pET23b vectors encoding different forms of p14ARF. Bacteria were grown, and protein expression was induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside for up to 16 h. Bacteria were sedimented and resuspended in denaturing buffer (6 M guanidinium-HCl, 150 mM NaCl, and 50 mM HEPES, pH 7.2), and then sonicated. Insoluble material was removed by centrifugation at 11,000 × g for 30 min. Imidazole was added to the solubilized protein to a concentration of 10 mM and then passed through a 2-ml nickel-nitrilotriacetic acid-Sepharose column equilibrated with the denaturing buffer. The column was then washed with 20 ml of wash buffer (8 M urea, 150 mM NaCl, 50 mM HEPES, pH 7.2), followed by 10 ml with MOPS buffer and 15 ml imidazole. His-tagged protein was eluted in wash buffer with 300 mM imidazole. Protein was dialyzed extensively against Tris-HCl, pH 7.5, 150 mM NaCl.

Gene Filtration Chromatography—Cell pellets were lysed in RIPA buffer (see above) and diluted with 4 volumes of Nonidet P-40 buffer. Lysates were centrifuged at 16,000 × g for 20 min, and supernatants were filtered through a 0.2-μm membrane. Total protein concentration was ~1 mg/ml as determined by Bradford assay. Alternatively, 12.5 μg of recombinant p14ARFC15,100,123A were diluted in 50 μl of the indicated buffer (RIPA/Nonidet P-40, 1:4) and filtered as above. 50 μl of sample (cell lysate or purified protein) were loaded onto a Superdex™ 200HR gel filtration column and separated using the Amersham Biosciences system. Fucose-labeled and unmodified p14ARF were performed in the same buffer used for the dilution of the samples. 50-μl fractions were collected. Bio-Rad Gel Filtration Standards were used for calibration.
slower mobility are also detected (Fig. 1A, right panel). The presence of the higher molecular mass forms of p14ARF was insensitive to the sulphydryl chelating agent N-ethylmaleimide in the lysis buffer. This result indicated that the higher molecular mass forms of p14ARF are produced inside the cell and not during the cell lysis preparation procedure.

To test whether the high molecular mass products actually contained the p14ARF moiety and were not due to the induction of the expression of proteins non-specifically recognized by the serum, we compared the mobility of full-length p14ARF (Fig. 1A, lanes 1 and 2) or full-length p14ARF (lanes 3 and 4). Cells were lysed in Nonidet P-40 buffer as described under “Materials and Methods” in the presence or absence of N-ethylmaleimide (200 mM), and the supernatant fractions were analyzed by SDS-PAGE in either non-reducing conditions (left panel) or in highly reducing conditions (right panel). Blots were developed using a rabbit serum against a carboxyl-terminal peptide of human ARF. The different bands recognized by this serum are indicated with an arrow.

Given the effect of reducing conditions on the electrophoretic mobility of p14ARF described above, we proceeded to mutate the cysteine residues to alanine. Mutation of single cysteine residues at the carboxyl terminus, cysteine 123 of the human p14ARF (p14ARF), is able to form homodimers inside the cell. An equivalent result was obtained when these experiments were carried out using the U2OS cell line, and therefore, our observations are not limited to a single type of cell (see Fig. 9C below). Supporting that p14ARF can oligomerize with itself, higher molecular weight forms of purified recombinant p14ARF could also be detected under non-reducing conditions (see below, Fig. 2C).

The Cysteine Residues in Human p14ARF Are Involved in the Stabilization of the Multimeric Forms of p14ARF—Unlike Mdm2 (27), ARF is poorly conserved between species. Only 38 of the 132 residues of human ARF are identical in both the mouse and opossum sequences (Fig. 2A). Human ARF (p14ARF) contains three cysteine residues at positions 15, 100, and 123 (Fig. 2A). Interestingly, the first two cysteine residues are adjacent to the arginine-rich sequences that are involved in the nucleolar localization of human ARF (12, 14). The cysteine residues at positions 15 and 100 of human ARF are conserved in the sequence of the opossum ARF protein but absent in the reported murine ARF sequence. The lack of conservation of the first cysteine residue in the mouse sequence is not surprising, because the nucleolar localization signal for the mouse ARF protein (p19ARF) has been mapped further downstream in the region between residues 26 and 37 (15). Coincidentally, there is a cysteine residue in the mouse sequence three residues downstream of the murine arginine-rich nucleolar localization signal. Although all three ARF sequences contain cysteine residues at the carboxyl terminus, cysteine 123 of the human p14ARF is only strictly conserved in mouse p19ARF.

We then tested whether the oligomerization of p14ARF described above, we proceeded to mutate the cysteine residues to alanine. Mutation of single cysteine residues still allowed the detection of the dimeric forms of p14ARF (Fig. 2B, lanes 2–4) and the substitution of cysteine 15 decreased the ability of p14ARF to form higher molecular mass complexes in SDS-gels (Fig. 2B, lane 2). Mutation of two of the three cysteines abolished the appearance of multimeric forms of p14ARF but did not affect the formation of p14ARF dimers (Fig. 2B, lanes 5–7). Finally, the appearance of the dimeric and higher molecular forms of p14ARF was markedly decreased when all three cysteines were mutated (Fig. 2B, lane 8). These results show that all three cysteine residues in p14ARF are involved in the formation or the stabilization of homodimers and higher order forms.

Recombinant p14ARF (His-tagged) analyzed in non-reducing conditions gave rise to the appearance of multimeric forms that were not present when the corresponding triple-cysteine mutant recombinant protein was analyzed (Fig. 2C). This supports that p14ARF can oligomerize with itself in the absence of other proteins.

Cysteine-dependent p14ARF Oligomerization Increases in the Presence of the Oxidants—We then tested whether the oligomerization status of p14ARF was sensitive to the redox status in the cell. Cells overexpressing p14ARF were treated with two oxidizing agents, hydrogen peroxide or the sulphydryl oxidizing agent azodicarboxylic acid bis(dimethylamide, diamide). As shown in Figs. 3 (A and B), after short incubations with
the cysteine residues in human p14ARF are involved in the appearance of multimeric forms of p14ARF. A, analysis of the cysteine residues in the ARF sequence. Alignment of the human, murine, and South American opossum ARF sequences. Identical residues in the three sequences are labeled with an asterisk. Cysteine residues are underlined. The arginine-rich nucleolar localization signals proposed for the human and mouse proteins (12, 14, 15) are boxed. B, comparison of the electrophoretic mobility of wild type p14ARF and p14ARF lacking cysteine residues. H1299 cells were transfected with an expression vector for wild type p14ARF (lane 1), p14ARF-C15A (lane 2), p14ARF-C100A (lane 3), p14ARF-C123A (lane 4), p14ARF-C15,100A (lane 5), p14ARF-C15,123A (lane 6), p14ARF-C100,123A (lane 7), p14ARF-C15,100,123A (lane 8), or pcDNA3 empty vector (lane 9). Cell pellets were lysed in Nonidet P-40 buffer, and the supernatants were analyzed by electrophoresis in non-reducing (left panel) and reducing (right panel) conditions and Western blotting using the rabbit polyclonal serum against p14ARF. C, purified recombinant His-tagged p14ARF (lanes 1) and p14ARF-C15,100,123A (lanes 2) were analyzed by electrophoresis in non-reducing conditions. In the left panel, proteins were detected by Coomassie Blue staining. In the right panel proteins were detected by Western blot using an antibody against the His tag.

These agents, the levels of high molecular mass forms of p14ARF increased in SDS-polyacrylamide gels, and a concomitant decrease in the levels of monomeric p14ARF was observed.

It must be stressed that in these and the previous experiments we were analyzing p14ARF in a soluble fraction prepared by lysing cells in mild conditions using the Nonidet P-40 buffer. Instead, most of p14ARF is in the insoluble fraction (Fig. 3C). Accordingly, extraction with harsher detergent conditions (SDS-PAGE loading buffer) dramatically increased the levels of p14ARF detected (Fig. 3C). Therefore, we also analyzed the effect of these oxidizing agents in whole cell extracts prepared by lysing the cells directly in SDS-PAGE loading buffer. As shown in Figs. 3C (left panel) and 3D, H2O2 and diamide have an effect on the total population of p14ARF by increasing the proportion of cysteine-linked oligomeric forms over the non-covalently linked forms of p14ARF. In Fig. 3C (right panel), it is shown that no increase in the oligomeric forms was detected with the triple-cysteine mutant of p14ARF (p14ARF-C15,100,123A) when cells were subjected to H2O2 treatment.

We also tested whether the appearance of the higher molecular mass forms after treatment of the cells with oxidizing conditions could be occurring during sample preparation. For this purpose we carried out a similar experiment as the one described in Fig. 1C. As shown in Fig. 3E, even in the presence of diamide, the intermediate form of p14ARF dimers (corresponding to p14ARF and p14ARF-D2–14 dimers) appeared only when the cells were cotransfected with p14ARF and the p14ARF-D2–14 deletion mutant but not when the cells expressing either full-length p14ARF or p14ARF-D2–14 were mixed. In the presence of diamide we could also observe an intermediate band in the 40-kDa region only when cells expressed both forms of p14ARF. This indicates that the bands appearing in this region correspond to trimeric forms of p14ARF. The nature of the upper bands was not investigated. Although the size of these higher bands is lower than what could be expected by the coupling of additional p14ARF molecules to the trimeric complex, it cannot be excluded that the electrophoretic migration of higher order complexes is anomalous.

We also analyzed the reversibility of the effect of H2O2 and diamide. As shown in Fig. 4 (A and B), the effect of these oxidizing agents was reversible within minutes of their removal from the cell culture medium.

Endogenous p14ARF Forms Stable Oligomers under Oxidizing Conditions—To rule out that the oligomerization of p14ARF was due to the overexpression of p14ARF we analyzed the oligomerization status of endogenous p14ARF in H1299 cells, which are known to express this protein at detectable levels (22). To reduce the nonspecific recognition of proteins by the rabbit serum against p14ARF we fractionated the cells and analyzed them by electrophoresis in non-reducing conditions and Western blotting. As shown in Fig. 5, in the fractions enriched in nucleoli, which is where p14ARF is primarily located, only the non-covalently linked form of p14ARF is detected. Instead, when the cells were treated with H2O2 and fractionated, the level of non-covalently linked p14ARF decreased and a novel band corresponding to the dimeric form of p14ARF was easily detectable (Fig. 5B). This indicates that, as with ectopic p14ARF, the proportion of covalently linked dimeric forms to non-covalently linked forms of endogenous p14ARF is increased when cells are incubated with H2O2. Therefore, we conclude that endogenous p14ARF can at least dimerize in response to oxidizing agents. Higher molecular mass p14ARF forms were also detected by the serum against p14ARF in the non-nucleolar fraction of the nucleus (Fig. 5). At
14. In all cases, cells were treated with 2 mM diamide for 30 min before lysis. In lane 2, the cells pellets analyzed in lanes 1, 4, and 7, treated with 0.2 mM H$_2$O$_2$ for 20 min (lanes 2, 5, and 8) or with 10 mM H$_2$O$_2$ for 30 min (lanes 3, 6, and 9). In lanes 1–6, cells were lysed in Nonidet P-40 buffer, and extracts were centrifuged. The supernatant-soluble fraction (lanes 4–6) and in the insoluble pellet (lanes 1–3) were analyzed. In lane 6–9, cells were lysed directly in SDS-PAGE loading buffer and total cell extracts were analyzed. Samples were separated by electrophoresis in non-reducing conditions and p14ARF by immunoblotting was detected as above. In the right panel the same experiment was carried out with cells transfected with the expression vector for p14ARF/15,100,123A. D, H1299 cells were transfected with the plasmid expressing wild type p14ARF and treated with 2 mM diamide for 0, 10, 20, and 30 min. Cells were lysed directly in SDS-PAGE loading buffer, and total cell extracts were analyzed for p14ARF expression. E, H1299 cells were transfected with either the full-length p14ARF expression construct (lane 1) or the construct expressing the p14ARFΔ2–14 deletion mutant (lane 3). In lane 2, the cells pellets analyzed in lanes 1 and 3 were mixed before lysis. In lane 4, the cells were transfected with the full-length p14ARF expression vector together with the construct expressing p14ARFΔ2–14. In all cases, cells were treated with 2 mM diamide for 30 min before harvesting. Cell extracts were prepared and electrophoresed in non-reducing conditions, and p14ARF was detected as above. Full-length p14ARF/p14ARFΔ2–14 dimers and trimers are indicated with arrows.

Fig. 3. Cysteine-dependent oligomerization of p14ARF increases with oxidizing agents. A and B, H1299 cells were transfected with the plasmid expressing wild type p14ARF and treated as indicated with H$_2$O$_2$ (A) or diamide (B). Soluble protein was extracted using the mild Nonidet P-40 buffer. C, in the left panel, H1299 cells were transfected with the plasmid expressing wild type p14ARF and left untreated (lanes 1, 4, and 7), treated with 0.2 mM H$_2$O$_2$ for 20 min (lanes 2, 5, and 8) or with 10 mM H$_2$O$_2$ for 30 min (lanes 3, 6, and 9). In lanes 1–6, cells were lysed in Nonidet P-40 buffer, and extracts were centrifuged. The supernatant-soluble fraction (lanes 1–3) and in the insoluble pellet (lanes 4–6) were analyzed. In lanes 6–9, cells were lysed directly in SDS-PAGE loading buffer and total cell extracts were analyzed. Samples were separated by electrophoresis in non-reducing conditions and p14ARF by immunoblotting was detected as above. In the right panel the same experiment was carried out with cells transfected with the expression vector for p14ARF/15,100,123A. D, H1299 cells were transfected with the plasmid expressing wild type p14ARF and treated with 2 mM diamide for 0, 10, 20, and 30 min. Cells were lysed directly in SDS-PAGE loading buffer, and total cell extracts were analyzed for p14ARF expression. E, H1299 cells were transfected with either the full-length p14ARF expression construct (lane 1) or the construct expressing the p14ARFΔ2–14 deletion mutant (lane 3). In lane 2, the cells pellets analyzed in lanes 1 and 3 were mixed before lysis. In lane 4, the cells were transfected with the full-length p14ARF expression vector together with the construct expressing p14ARFΔ2–14. In all cases, cells were treated with 2 mM diamide for 30 min before harvesting. Cell extracts were prepared and electrophoresed in non-reducing conditions, and p14ARF was detected as above. Full-length p14ARF/p14ARFΔ2–14 dimers and trimers are indicated with arrows.

Fig. 4. The effect of oxidizing agents on the oligomerization of p14ARF. A, H1299 cells were transfected with the plasmid expressing wild type p14ARF. In lane 1 cells were left untreated. In lanes 2–5, cells were treated with 20 μM H$_2$O$_2$ for 10 min after which they were changed to medium without H$_2$O$_2$ and analyzed at 0, 30, 60, and 90 min after removal (lanes 2–5, respectively). Cells were lysed by SDS-PAGE loading buffer, samples were separated in non-reducing conditions, and p14ARF was analyzed by immunoblotting. B, the same experiment was performed treating the cells with 2 mM diamide for 30 min.

Fig. 5. Endogenous p14ARF oligomerization is increased in response to oxidative stress. H1299 cells were left untreated (lanes 1–4) or treated with 10 mM H$_2$O$_2$ for 5 min (lanes 5–8). In lanes 9–12 cells were transfected with the p14ARF expression vector. Cells were fractionated as described under "Materials and Methods," and the cytoplasmic (lanes 1, 5, and 9), nuclear (lanes 2, 6, and 10), nuclear non-nucleolar (lanes 3, 7, and 11), and nucleolar (lanes 4, 8, and 12) compartments were analyzed for their p14ARF content by Western blotting in non-reducing conditions. The position of the p14ARF dimeric form is indicated by an asterisk. Detection of the nucleolar antigen B23 (nucleophosmin) protein and the proteasome core protein α6 were used to assess the quality of the fractions.

present we do not know whether these bands are specific, but they were not sensitive to the H$_2$O$_2$ treatment.

Binding of the Different Forms of p14ARF to Mdm2—The next question addressed was whether the disulfide-linked forms of p14ARF were able to bind to Mdm2. Both wild type p14ARF and the triple-cysteine mutant co-immunoprecipitated with Mdm2 and in the conditions tested, and the binding of the wild type p14ARF was not affected by the addition of H$_2$O$_2$ (Fig. 6A). This indicated that the oligomeric forms of p14ARF can bind to hMdm2. To confirm this, we analyzed the p14ARF bound to hMdm2 in reducing and non-reducing conditions (Fig. 6B). When the samples were electrophoresed in non-reducing conditions, although the p14ARF triple-cysteine mutant could be effectively detected, the monomeric form of wild type p14ARF was markedly reduced and the higher molecular mass forms were difficult to distinguish from the background immunoglobulin chains. However, when the same samples were analyzed in reducing conditions, wild type p14ARF could be detected as efficiently as the triple-cysteine mutant. This shows that Mdm2 can interact with the covalently-linked forms of p14ARF in cell extracts.

However, it was still conceivable that p14ARF binds to Mdm2 preferentially when it is not covalently linked and that, once p14ARF and Mdm2 are bound, covalent oligomerization of p14ARF can take place. To test this possibility p14ARF and p14ARFC15,100,123A were expressed by in vitro transcription translation in the presence of [35S]methionine and analyzed by electrophoresis in non-reducing conditions. As shown in Fig. 6C, a large proportion of p14ARF but not p14ARFC15,100,123A migrated to positions that correspond to its dimeric and multimeric forms. In Fig. 6D, recombinant human Mdm2 (hMdm2) was added to [35S]-labeled p14ARF and then Mdm2 was immunoprecipitated. When the samples were
analyzed by electrophoresis in non-reducing conditions only the monomeric form of wild type p14ARF but not its covalently linked forms interacted with hMdm2 in these conditions. This result suggests that the covalently linked forms of p14ARF have a lower affinity for Mdm2, indicating that the regions of p14ARF necessary for its interaction with hMdm2 are not accessible in the high molecular weight complexes.

Analysis of the Function of the p14ARF Triple Cysteine Mutant—As shown in Fig. 7A, the triple-cysteine mutant of p14ARF is not defective for nucleolar localization. Furthermore, and like wild type p14ARF (11), this mutant protected p53 from Mdm2-mediated degradation and increased the levels of exogenous Mdm2 in a p53 negative background (Fig. 7, A and B). Accordingly, the triple-cysteine mutant was active in increasing the transcriptional activity of endogenous p53, as determined using a p53 reporter construct in the p53 positive U2OS cells (Fig. 7, A and C). Interestingly, the control SV promoter-driven luciferase activity was consistently lower in U2OS cells (Fig. 7, A and B).

The Region between Residues 2 and 14 of p14ARF Impairs the Formation of Highly Stable Oligomeric Forms—We observed that unlike full-length p14ARF, at least 50% of the total p14ARF32–14 deletion mutant forms remarkably stable oligomers in H1299 cells, even when it is subjected to denaturing electrophoresis in reducing conditions (Fig. 8A, lane 4). Furthermore, when the three cysteine residues of this mutant were substituted by alanine, dimeric forms of the deleted protein were still observed after electrophoresis (Fig. 8B). Addition of 8 M urea in the sample buffer had no effect on the pattern of bands (Fig. 8B). Because the proportion of the higher molecular mass forms is significantly higher with the p14ARF32–14 than with the full-length p14ARF (Fig. 8A, lanes 1 and 3), we conclude that the formation of these structures is impaired by the presence of the first 14 amino acids in p14ARF. Accordingly, a mutation of the conserved threonine residue at position 8 of p14ARF to aspartic acid increased the proportion of the oligomeric forms in the presence of reducing agents (Fig. 8A, lane 3). Again, the multimeric forms of this mutant were still detected after mutation of all of its cysteine residues (Fig. 8A, lanes 3 and 7). Interestingly, this mutant migrated faster than other full-length forms of p14ARF.

p14ARF Can Form High Molecular Weight Complexes Even in the Absence of Cysteines—The results obtained in the previous section raised the possibility that full-length p14ARF could form stable oligomers even in the absence of cysteine residues. When the membranes are overexposed as in Fig. 8, the dimeric forms of the p14ARF triple-cysteine mutant of full-length p14ARF appear even in reducing electrophoresis conditions (Fig. 8A, lane 6). Accordingly, we observed that the p14ARF32–14 mutant could form oligomers with the triple full-length cysteine mutant p14ARFC15,100,123A (Fig. 8D). Furthermore, p14ARF14–2–14 was still able to oligomerize with p14ARFC15,100,123A, although the proportion of stable oligomeric forms is significantly lower. Although the cells used for this assay (U2OS) have been described as negative for p14ARF expression (22) low levels of a band that could correspond to endogenous p14ARF were detected. However the presence of this band did not justify the appearance of p14ARFC15,100,123A–p14ARF2–14 heterodimers.

Therefore, we tested the oligomerization status of the triple-cysteine mutant of p14ARF in conditions that are milder than the separation of proteins by denaturing electrophoresis. U2OS cells were transfected with the p14ARFC15,100,123A expression vector, lysed in RIPA buffer (containing 0.1% SDS). Extracts were diluted in Nonidet P-40 buffer, the insoluble fraction was removed by centrifugation, and the remaining supernatant was filtered through a membrane (pore size, 0.2 μm). These two steps rule out the existence of intact nucleioli in the samples. As shown in Fig. 9A, when samples were subjected to gel filtration chromatography, all of the p14ARF in the soluble extract was exclusively detected in a single broad peak that corresponds to the elution of proteins with a molecular mass above the 670-kDa marker. We also carried out the same experiment analyzing ectopic wild type p14ARF as well as the endogenous p14ARF in H1299 cells. As shown in Fig. 5, endogenous p14ARF is found in a non-covalently linked form under normal growth conditions. However, most of this protein eluted by gel filtration also formed very high molecular weight complexes that eluted in the same fractions as p14ARFC15,100,123A (Fig. 5B). Interestingly, unlike ectopic wild type p14ARF or p14ARFC15,100,123A, a significant proportion of the endogenous p14ARF eluted in fractions with an apparent molecular mass of 158 kDa. It must be stressed here that the size of these complexes is dramatically higher than the molecular weight expected for an Mdm2:p14ARF complex. Ec-
Fig. 7. Functional analysis of the p14ARF triple-cysteine mutant. A, U2OS cells were transfected with plasmids expressing wild type p14ARF or p14ARFC15,100,123A (p14ARFCysmt). Cells were fixed and stained with the rabbit serum against p14ARF. The percentage of p14ARF-positive cells with nucleolar staining (n), nucleolar and nucleoplasmic staining (nN), or non-nucleolar staining (non-n) were scored. At least 850 cells were counted in each case. B, H1299 cells were transfected with expression vectors for p53, murine Mdm2, p14ARF, and p14ARFC15,100,123A as indicated. p53 was detected by Western blot using the DO1 mouse monoclonal antibody. C, U2OS cells were transfected with a lacZ reporter plasmid driven by the RGC4fos-p53-dependent promoter and a luciferase reporter plasmid driven by the SV promoter in the presence of 6.25 ng of control pcDNA3, pcDNA3p14ARF, or pcDNA3p14ARFC15,100,123A. p53-dependent lacZ expression (top panel) and SV-driven luciferase activity (lower panel) were analyzed after 48 h. D, U2OS cells were transfected with 1 μg of empty pcDNA3 vector or the plasmids expressing the indicated proteins and analyzed by clonogenic assay. A representative experiment is shown. E, quantification of the growth inhibitory effects of p14ARF and p14ARFC15,100,123A on U2OS cells by clonogenic assay. The number of colonies in each plate was divided by the number of colonies obtained with the empty pcDNA3 vector and expressed as a percentage. The average and standard deviation of four experiments performed using 100 and 10 ng of each DNA is shown.

topic wild type p14ARF had the same elution pattern as p14ARFΔ2–14 (Fig. 9C).

This result indicates that p14ARF monomers may interact with other components that form part of stable and very abundant high molecular weight soluble complexes. The nucleolar protein fibrillarin could be detected in the same fractions as p14ARF (Fig. 9C), and therefore we cannot rule out that monomeric p14ARF could be associated with nucleolar fragments in the filtered extracts. Another nucleolar protein B23, which is known to form high molecular weight homo-oligomers (28, 29) in p14ARF. Interestingly, disulfide bridges are not important for p14ARF can form large complexes in cells and that the formation of these complexes does not require the cysteine residues in p14ARF. Instead, one or more carboxyl-terminal fragments of less than 14 kDa was detected in fractions 23 through 29. Small amounts of a small carboxyl-terminal fragment could also be detected in the input protein. Interestingly, fractions 28 and 29 also contained a slower mobility form. Supposing that this carboxyl-terminal fragment behaves in a similar way as the p14ARFΔ2–14 deletion mutant, this slower mobility form could correspond to dimers of this small carboxyl-terminal fragment formed after elution from the column. When the purified protein sample was diluted and filtered in the presence of 1 mM MgCl2 not even the smaller carboxyl-terminal fragment of p14ARF was detected (not shown). It is interesting to notice here that divalent metal ions promote the formation of large B23 complexes (29). In the presence of recombinant hMdm2, neither p14ARFC15,100,123A nor hMdm2 were detected in any of the fractions (not shown). This is in agreement with the observation that incubation of a 37-residue amino-terminal fragment of p14ARF together with the p14ARF-binding domain of hMdm2 induces the appearance of large extended structures that elute in the void volume of gel filtration columns (30).

Taken together, these results agree with the hypothesis that p14ARF can form large complexes in cells and that the formation of these complexes does not require the cysteine residues in p14ARF. Interestingly, disulfide bridges are not important in B23 oligomer formation (29).

Because denaturing gel electrophoresis conditions are too harsh to evaluate the existence of non-covalently linked p14ARF complexes, and gel filtration analysis did not allow us to unequivocally establish that p14ARF can form non-covalently linked oligomers, we took a different approach based on the impaired ability of the p14ARFΔ2–14 mutant to localize to the nucleolus (31). His-tagged full-length wild type p14ARF or p14ARFC15,100,123A were overexpressed with or without the p14ARFΔ2–14 or the p14ARFΔ2–14 C15,100,123A mutants, respectively, and the percentage of cells with full-length p14ARF in the nucleolus was evaluated using an antibody against the histidine tag. As shown in Fig. 10, the presence of
FIG. 8. p14ARF oligomerizes independently of its cysteine residues. A, H1299 cells were transfected with the control vector pCDNA3 (lane 1) or expression vectors for p14ARF (lane 2), p14ARF T8D (lane 3), p14ARFΔ2–14 (lane 4), p14ARFΔ382–101 (lane 5), p14ARFΔC15,100,123A (lane 6), or p14ARFΔC15,100,123A (lane 7). Cell extracts were subjected to SDS-PAGE electrophoresis in the presence of reducing agents, and the different p14ARF forms were analyzed by Western blotting. B, H1299 cells were transfected with p14ARFΔ2–14 (lanes 1 and 2) or p14ARFΔ2–14ΔC15,100,123A (lanes 3 and 4). In lanes 2 and 4, lysates were treated with 8 M urea. Cell extracts were subjected to SDS-PAGE electrophoresis in non-reducing (left panel) or reducing (right panel) conditions, and the different p14ARF forms were analyzed by Western blotting. U2OS cells were transfected with expression vectors for p14ARFΔ2–14 (lane 1), p14ARFΔC15,100,123A (lane 2), p14ARFΔ2–14 and p14ARFΔC15,100,123A (lane 3), p14ARFΔ2–14 and p14ARF (lane 4), or p14ARF (lane 5). Cell extracts were subjected to SDS-PAGE electrophoresis in the absence of reducing agents, and the different p14ARF forms were analyzed by Western blotting. The different dimeric forms are indicated by arrows. Low levels of a band with a slightly slower mobility than monomeric full-length p14ARF were detected. Whether this band corresponds to endogenous p14ARF has not been established. This band is indicated with a gray arrow.

the p14ARFΔ2–14 clearly decreased the number of cells with full-length p14ARF in the nucleus. Supporting that p14ARF can form homo-oligomers even in the absence of cysteines, the triple-cysteine mutant of p14ARFΔ2–14, also displaced the triple-cysteine mutants of full-length His-tagged p14ARF from the nucleus.

DISCUSSION

Several pathological processes related to aging are associated with an imbalance of the cellular redox status to a more oxidant state (reviewed in Ref. 32). Tumor promotion has also been associated with an altered redox status, and it is known that cancer cells produce an excess of reactive oxygen species. Oxidants are generated as a result of normal metabolism, and their levels are further increased with aging due to leakage in electron transport and less efficient antioxidant systems. In addition, a number of agents (UV and ionizing radiation, chemotherapeutics, and environmental toxins) can trigger the production of reactive oxygen species. The results presented here may suggest that the response of cells to antitumor therapies may be influenced by the effects of oxidative stress on p14ARF and that p14ARF function may be affected by aging.

We have presented data strongly suggesting that human ARF is able to oligomerize and that the detection of its oligomeric forms is sensitive to reducing agents during electrophoresis. Accordingly, we have shown that all three cysteines in p14ARF are involved in the stabilization of the oligomers. Although p14ARF can form homodimers and homotrimers, this does not exclude the possibility that p14ARF may also interact with other proteins in a cysteine-dependent manner. In this sense it is worth noticing that there is a very complex pattern of other higher molecular mass forms that we have not analyzed.

The triple-cysteine mutant of p14ARF is normal in its localization and is fully active and reproducibly more active than wild type p14ARF in its p53-related functions. Whether this slightly higher activity of the p14ARF triple-cysteine mutant could be related to the higher expression levels of this protein or to an improvement of its activity was not possible to determine. In all cases, the levels of the cysteine mutant were either higher or the same as the levels of wild type ARF, but never lower. The stronger activity of the p14ARF triple-cysteine mutant could also be related to the lack of binding of the cysteine-linked forms of p14ARF to Mdm2 as shown in vitro. However, this was not possible to establish, because in cell extracts, the cysteine-linked forms of p14ARF co-immunoprecipitated with Mdm2.

Oligomerization can be detected in normal growth conditions and dramatically increases when cells are treated with oxidizing agents. It is important to notice that the effect of oxidative agents was also detected with endogenous p14ARF, implying that our observations are not simply due to unspecific aggregation of the protein when it is overexpressed.

We also analyzed whether p14ARF could form non-covalently linked oligomers in normal conditions. Supporting the existence of non-covalently linked oligomeric forms of p14ARF, a small proportion of full-length p14ARF triple-cysteine mutant could be detected as highly stable dimers. This is further strengthened by the alteration of the subcellular localization of the full-length triple-cysteine mutant by p14ARFΔ2–14. Finally, gel filtration analysis of the p14ARF triple-cysteine mutant expressed in cells and of the purified recombinant protein do not disagree with the hypothesis that p14ARF can form high molecular weight complexes that are not linked by covalent bonds. A similar behavior has been described for B23 (28, 29) a nucleolar protein that, like p14ARF, is able to stabilize and induce the activity of the p53 tumor suppressor (33). The possibility that p14ARF can oligomerize even in the absence of cysteines may help to explain the lack of dramatic differences between the function of p14ARF and p14ARFΔC15,100,123A in normal growth conditions. We have also tested whether the functions of wild type p14ARF and p14ARFΔC15,100,123A differed in the presence of the oxidizing agents diamide and H₂O₂, but no reproducible differences, even within the same experiment, were observed in the conditions tested (not shown). It must be stressed that the addition of these oxidizing agents is highly toxic and is bound to have pleiotropic effects that may not allow the detection of differences specifically due to p14ARF. This problem may be solved using stable cell lines expressing the different forms of p14ARF under an inducible promoter.

A mutant of p14ARF lacking residues 2–14 forms extremely stable oligomers even when all of its cysteines were mutated. This was also observed with the full-length p14ARF with the TSD point mutation in this region. Because the proportion of these complexes is not so high with all forms of p14ARF that have an intact amino terminus, we conclude that the amino-terminal 14 residues of p14ARF impair the formation of multi-meric hyper-stable complexes.

The first 37 amino acids of p14ARF are sufficient for Mdm2 binding and p53 stabilization (31, 34). In particular, the first 14
amino acids of p14ARF have been shown to be very important for the ability of p14ARF to protect p53 from Mdm2-mediated degradation (11, 31). Accordingly, peptides containing the 15 first amino acids of p14ARF bind to Mdm2 in vitro (10, 30, 35). However, as reported by Bothner and co-workers (30) overlapping peptides spanning regions 11–25 and 16–30, at least in the conditions tested by these authors, can also bind to Mdm2. Accordingly, the work of Clark and co-workers (17) shows that multiple domains in the first 46 amino acids of p14ARF are involved in its effects in vivo. Strikingly, in their conditions, the deletion of amino acids 2–10 and even 2–20, only slightly impaired the function of p14ARF in their assays. The situation becomes even more complex when the murine version of p14ARF, p19ARF, is analyzed (18, 30).

All of these results indicate that at least two regions in the exon 1-encoded fragment of p14ARF are important for its functionality and that they may bind to Mdm2 with different affinities. These two regions were proposed to locate between residues 3 and 10 and residues 21 and 29 of both p14ARF and p19ARF, two segments that share striking sequence similarity with each other (30). According to the results of DiGiammarino et al. (34) a soluble form of the first 37 amino acids of murine p19ARF is highly unstructured in aqueous conditions whereas in a non-aqueous environment, the 4–14 and 20–29 regions acquire an α-helix conformation. Furthermore, in the presence of Mdm2, these two regions adopt a β-strand conformation (30), and, in this situation, large complexes are formed. As discussed by Bothner and colleagues (30) the ARF/Mdm2 system is similar to amyloid proteins in that they form extended networks comprised of β-strands. This effect may be related to our observations on the oligomerization of full-length p14ARF in vivo.

In summary, mapping of the active regions of ARF has

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**Fig. 9.** A, 3 × 10⁵ U2OS cells were seeded and transfected with the expression vector for p14ARFC15,100,123A. Cells were harvested and lysed in 50 μl of RIPA buffer and diluted with 200 μl of Nonidet P-40 buffer. After centrifugation and filtration of the supernatant through a 0.2-μm membrane, the proteins were separated using a Superdex 200 column and analyzed by Western blot in reducing conditions. The fraction numbers and the position where molecular mass markers elute are indicated at the top. In the top panel, p14ARFC15,100,123A was analyzed. In the lower panel, the B23 protein is detected. B, 3 × 10⁵ H1299 cells were seeded and lysed as above, and supernatants were subjected to gel filtration and Western blotting in reducing conditions. Endogenous p14ARF and fibrillarin were detected in the top and lower panels, respectively. Samples were analyzed in reducing conditions. C, gel filtration analysis was carried out with extracts of H1299 cells transfected with the vector expressing wild type p14ARF. In this case, samples were analyzed in non-reducing conditions. D, p14ARFC15,100,123A was subjected to gel filtration in the RIPA buffer:Nonidet P-40, 1:4 (top panel), or Tris-HCl/150 mM NaCl, pH 7.5 (lower panel). Samples were analyzed in reducing conditions.

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**Fig. 10.** 10⁵ U2OS cells were transfected with the indicated amounts of pcDNA3 vectors expressing wild type His-tagged p14ARF (p14ARF-His) or its triple-cysteine mutant form (p14ARFCysmt-His) in the absence or presence of 1 μg of p14ARFΔ2–14 expression vector. After 36 h, cells were co-stained with the rabbit serum against the carboxyl terminus of p14ARF and a mouse monoclonal antibody against the His tag. The ratio between the number of cells positive for nucleolar staining with the anti-His antibody and the number of cells positive for the p14ARF serum irrespective of localization is expressed as a percentage.
proven to be a difficult task. This may have several causes: 1) Several active regions (17, 18) may act in an additive fashion. 2) Two of these regions have similar sequence features and may therefore, to some extent, substitute for each other. 3) According to the observations made by DiGiammarino et al. (34) and Bothner et al. (30) ARF could exist in different conformations in different conditions. 4) Adding to this complexity, the results presented here suggest that p14ARF can form oligomeric complexes. As mentioned above, the p14ARF protein may be non-accessible in the high molecular weight complexes. 5) Whether p14ARF oligomerization or conformational changes are functionally important needs further analyses. Both mapping of the one or more regions in p14ARF that mediate oligomerization and the study of whether the long term response of cells and animal models to oxidative stress affects ARF oligomerization and/or is dependent on ARF will be essential. Finally, our observations may help in the design of effective p14ARF peptide mimics leading to the development of novel non-genotoxic antitumor treatments and may be essential to consider for in vitro studies using purified protein.

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Oligomerization of the Human ARF Tumor Suppressor and Its Response to Oxidative Stress

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