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ARF Function Does Not Require p53 Stabilization or Mdm2 Relocalization

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It is generally accepted that the ARF tumor suppressor induces p53-dependent growth arrest by sequestering the p53 antagonist Mdm2 in the nucleolus. Previous mutagenic studies of murine ARF suggested that residues 1 through 14 and 26 through 37 were critical for Mdm2 binding, while the latter domain also governed ARF nucleolar localization. We show that mouse ARF residues 6 to 10 and 21 to 25 are required for ARF-induced growth arrest whereas residues 1 to 5 and 29 to 34 are dispensable. Deletion of the putative nucleolar localization signal ^{31}RRPR^{34} did not prevent nucleolar localization. Surprisingly, unlike wild-type ARF, growth-inhibitory mutants D1–5 and D29–34 failed to stabilize p53 yet induced its transcriptional activation in reporter assays. This suggests that p53 stabilization is not essential for ARF-mediated activation of p53. Like wild-type ARF, both mutants also exhibited p53-independent function since they were able to arrest *p53*/*Mdm2*-null cells. Notably, other mutants lacking conserved residues 6 to 10 or 21 to 25 were unable to suppress growth in p53-positive cells despite nucleolar localization and the ability to import Mdm2. Those observations stood in apparent contrast to the ability of wild-type ARF to block growth in some cells without relocalizing endogenous Mdm2 to nucleoli. Together, these data show a lack of correlation between ARF activity and Mdm2 relocalization, suggesting that additional events other than Mdm2 import are required for ARF function.

The alternative reading frame product, ARF, is derived from the *INK4a*/*ARF* tumor suppressor locus (54). The *INK4a*/*ARF* gene utilizes overlapping reading frames within its second exon to generate two unrelated growth inhibitors, p16^{INK4a} and p19^{ARF} (47). p16^{INK4a} functions in the retinoblastoma (pRb) tumor suppressor pathway (4, 52), whereas ARF protects against aberrant cell growth by activating the p53 tumor suppressor protein (54). p53 is a transcription factor that maintains genomic stability in response to DNA damage, hypoxia, oncogenic insults, and other cellular stresses (29, 31). Genotoxic stress rapidly stabilizes and activates p53 through post-transcriptional mechanisms (1, 15), enabling p53 to transactivate genes that trigger growth arrest or apoptosis (31). Together, *p53* and *INK4a*/*ARF* represent the two most frequently inactivated genes in human cancer (17, 50).

ARF is a key mediator of p53-dependent growth suppression in response to activated oncogenes. In normal cells, oncogenic Ras (44, 53), c-Myc (66), adenovirus E1A (6), E2F-1 (8), and v-Abl (5) induced ARF expression and consequent p53-mediated cell death or growth arrest. Conversely, in *ARF*-null cells, the same stimuli failed to activate p53 and those cells became rapidly transformed (5, 6, 28, 66). These findings were consistent with the ability of ARF to induce G_1 and G_2 phase growth arrest when overexpressed in p53-positive primary cells and tumor-derived cell lines, whereas it had no antiproliferative activity in p53-null cells (26, 55). An ARF-p53 pathway was further suggested by observations that ablation of either gene in mice had similar biological consequences. Both types of animals developed spontaneous tumors at an early age, and embryo fibroblasts derived from *ARF*- or *p53*-deficient mice failed to undergo senescence in culture (9, 18, 26).

ARF is thought to activate p53 by neutralizing the activities of the Mdm2 oncoprotein. As a transcriptional target of p53, Mdm2 participates in an autoregulatory feedback loop to antagonize p53 function (41). Mdm2 binds to p53 and blocks its transcriptional activity (42, 43), acts as an E3 ubiquitin ligase to target p53 for degradation in cytoplasmic proteasomes (19, 20, 30), and accelerates p53 nuclear export (13, 49, 58). Mdm2 and p53 are nucleoplasmic proteins, while ARF resides within nucleoli (54). The current view of ARF function is that it sequesters Mdm2 in nucleoli, thereby allowing p53 to accumulate in the nucleoplasm and induce the expression of growth-inhibitory genes (54). This model reflects studies showing that ARF can bind to Mdm2 (25, 45, 64), relocalize it to nucleoli (35, 36, 61, 62), and block Mdm2-mediated ubiquitination and nuclear export of p53 (21, 57). ARF mutants that failed to localize to nucleoli, or did not import Mdm2, were unable to stabilize p53 and were inactive in suppressing growth (61, 62). The essential functional domains identified within mouse ARF were residues 1 through 14 and 26 through 37. Both regions formed cooperative binding sites with Mdm2, while the latter encompassed a putative nucleolar localization signal (residues ^{31}RRPR^{34}) (61, 62). In apparent opposition to the above findings, others recently demonstrated that ARF could inhibit growth without relocalization of endogenous Mdm2 to nucleoli (32, 34) and that nucleoplasmic forms of ARF could stabilize p53 (34).

Importantly, ARF can also inhibit growth through p53- and Mdm2-independent mechanisms which are presently undefined. This was first indicated by studies showing that various types of human tumors exhibit simultaneous loss of functional

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p53 and ARF (11, 33, 40, 51), that mouse B-cell lymphomas lacking both proteins grow faster than those lacking p53 or ARF alone (10), and that some tumors which overexpress Mdm2 sustain deletion of *ARF* (10). These results suggested nonoverlapping functions for ARF, p53, and Mdm2. Indeed, mice lacking both ARF and p53 developed multiple primary tumors of a wider variety than did animals lacking either tumor suppressor alone (60). Moreover, ARF was able to inhibit the growth of *p53*/*Mdm2*-null cells (60). Since ARF has no growth-inhibitory activity in p53-negative cells, the latter result indicated that ARF can function independently of p53 when Mdm2 is also eliminated. Most significantly, those experiments revealed that other mediators of ARF-induced growth suppression exist besides Mdm2 and p53.

In this study, we generated amino-terminal deletion mutants of murine ARF to identify domains required for its nucleolar localization, regulation of Mdm2 and p53, and growth-suppressive activity. We identified two ARF mutants that were unable to suppress growth despite nucleolar localization and ability to import Mdm2, whereas other ARF mutants blocked growth without stabilizing p53. Moreover, as recently reported by others (32, 34), we showed that wild-type ARF can inhibit growth without relocalizing endogenous Mdm2. These results show that ARF function does not require p53 stabilization or Mdm2 import and suggest that additional factors mediate ARF-induced growth arrest.

MATERIALS AND METHODS

Cell culture and protein expression. Murine NIH 3T3 fibroblasts and human U2OS osteosarcoma cells (both *ARF*-null, *p53* and *Mdm2* wild type), and a U2OS derivative, NARF6 cells (kindly provided by Gordon Peters, Imperial Cancer Research Fund), were maintained in Dulbecco modified Eagle medium containing 10% fetal bovine serum, 2 mM glutamine, and 100 μg of penicillin and streptomycin per ml. Primary mouse embryo fibroblasts (MEFs) lacking p53, Mdm2, and ARF (kindly provided by Gerry Zambetti, St. Jude Children's Research Hospital) (60) were grown in the same medium supplemented with 0.1 mM nonessential amino acids and 55 μM 2-mercaptoethanol. Retroviral production and infections were performed using pSRα-MSV-tkCD8 or pSRα-MSV-tkneo plasmids containing hemagglutinin (HA)-tagged wild-type ARF or its mutants, as described previously (46, 47). Cells were transfected using Lipofectamine (Gibco-BRL), as specified by the manufacturer, or by a modified calcium phosphate precipitation (2).

Construction of ARF mutants. Deletion mutants of murine $p19^{ARF}$ were generated by PCR using a HA-tagged ARF cDNA template (47). Mutated sense and antisense oligonucleotides complementary to the noncontiguous sequences flanking the intended deletion site were used in two sequential reactions. Sense primers for D1-5 (5′-CTGACCGGTTTGGTCACTGTGAGGATTCA-3′), D6-10 (5′-CCGGTCGCAGGTTCATTCAGCGCGCGGG-3′), D21-25 (5′-GCCCA CTCCAAGAGAAGTTCGTGCGATC-3′), or D29-34 (5′-TGGTGAAGTTCG TGACAGCGAGCTGCGC-3′) were mixed with a T3 primer. Antisense primers for D6-10 (5′-CCCGCGCGCTGAATGAACCTGCGACCGG-3′), D21-25 (5′-GATCGCACGAACTTCTCTTGGAGTGGGC-3′), or D29-34 (5′-GCGCAGC TCGCTGTCACGAACTTCACCA-3′) were mixed with a T7 primer. PCR was carried out under standard conditions as described previously (46), purified PCR products for each internal deletion mutant were mixed, and PCR was repeated with outer T3 and T7 primers to obtain full-length mutants. Products were directly ligated into pCR-Script vectors for sequencing and then shuttled into pcDNA3 or pSRα-MSV-tk-CD8 and pSRα-MSV-tk-neo retroviral expression plasmids (46, 47). Previously characterized HA-tagged ARF constructs used as controls in these studies (pSRα-MSV-tk-neo plasmids containing D1–14, D26–37, and double mutant D1–14;D26–37) were graciously provided by Chuck Sherr, St. Jude Children's Research Hospital (61, 62).

Analyses for growth arrest. Cell cycle distributions of ARF-transduced cells were determined 60 to 72 h postinfection by dual-color flow cytometry of fixed cells that were doubly stained with propidium iodide and a CD8 antibody coupled to fluorescein isothiocyanate (FITC) (37). Samples were analyzed on a FACScan instrument (Becton Dickinson), and the cell cycle distributions of CD8-positive and -negative cells were determined using ModFit (Verity software).

Cell cycle progression into S phase was also monitored by bromodeoxyuridine (BrdU) incorporation into newly synthesized DNA. NIH 3T3, U2OS, or *p53*/*Mdm2*/*ARF*-null fibroblasts were seeded onto eight-well poly-L-lysine-coated chamber slides (1.5×10^4 cells/well) and infected with retroviruses, and 10 μM BrdU was added to the culture medium 24 h before fixation. The cells were fixed for 10 min at −20°C in methanol-acetone (1:1) and stained for 1 h with affinity-purified rabbit antisera to mouse ARF (47), followed by biotinylated anti-rabbit immunoglobulin and streptavidin-conjugated Texas red (Amersham). The cells were treated with 1.5 N HCl for 10 min and stained with a mouse monoclonal antibody to BrdU (Amersham) for 1 h, and BrdU incorporation was detected with a FITC-conjugated anti-mouse immunoglobulin (Amersham). Cells were visualized by confocal microscopy.

Immunoprecipitation and Western blot analyses. Proteins were released from whole cells by lysis (10^7 cells per ml) of frozen cell pellets for 1 h on ice in NP-40 buffer (50 mM Tris [pH 7.5], 120 mM NaCl, 1 mM EDTA, 0.5% NP-40) supplemented with 0.1 mM sodium vanadate, 1 mM sodium fluoride, 5 μg of leupeptin per ml, and 30 μM phenylmethylsulfonyl fluoride. After occassional vortexing and brief sonication (two 5-s pulses), lysates were clarified by centrifugation at 12,000 rpm for 10 min at 4°C. Equivalent amounts of proteins (300 μg per sample) were immunoprecipitated with protein A- or G-Sepharose at 4°C using antibodies to ARF (47) or Mdm2 (2A10 hybridoma supernatant [generously provided by Arnold Levine, Rockefeller University, and by Larry Zambetti] or SMP-14 [Oncogene Sciences]). Immune complexes, as well as lysates for direct Western blotting (50 μg of protein per lane), were separated on denaturing gels and transferred by electroblotting onto polyvinylidene difluoride membranes (Millipore). Proteins were detected by enhanced chemiluminescence (Amersham) as specified by the manufacturer using affinity-purified antisera to ARF (47), cyclin G1 (Santa Cruz), and p53 (Oncogene Sciences). Mdm2 was detected using 2A10 hybridoma supernatant at a 1:200 dilution in block solution (Tris-buffered saline containing 0.1% Tween 20 and 5% nonfat dried milk).

Localization assays. NIH 3T3 cells (2×10^5) were seeded onto glass coverslips and transfected using Lipofectamine or calcium phosphate precipitation with pcDNA3 plasmids encoding ARF or its mutants together with wild-type Mdm2. Human U2OS cells were similarly transfected with ARF constructs, while NARF6 cells were treated with 1 mM isopropyl β-D-thiogalactopyranoside (IPTG). Cells were fixed 48 h later with methanol-acetone (1:1) and stained for 1 h with affinity-purified rabbit antisera to mouse (10 μg/ml) or human (1:100 dilution; Novus Biologicals) ARF. Samples were incubated with biotinylated anti-rabbit immunoglobulin (1:500 dilution) and streptavidin-conjugated Texas Red (1:200 dilution; Amersham), as described previously (47). Mdm2 was detected using SMP14 monoclonal antibody (1:100 dilution; Santa Cruz Biotechnology) followed by fluorescein isothiocyanate (FITC)-conjugated anti-mouse immunoglobulin G (1:500 dilution; Amersham). To identify nucleoli, fixed cells were stained with antibodies to fibrillarin (1:5 dilution, ANA-N positive control; Sigma) for 1 h and then incubated for 30 min with FITC-conjugated anti-human immunoglobulin G (Amersham).

p53 reporter assays. G418-resistant clones of NIH 3T3 cells were established that stably expressed the p53 luciferase reporter construct, p53-luc (Stratagene), kindly provided by Stefan Ries and Frank McCormick (University of California San Francisco). The resulting 3T3-p53 reporter cell lines were infected with pSRα-MSV-tkneo plasmids containing HA-tagged wild-type ARF or its mutants, as described above. At 2 days after infection, cells were lysed and samples were measured in triplicate for luciferase activity as specified by the manufacturer (Promega luciferase assay system). The same populations of infected cells were simultaneously examined by ARF-BrdU immunofluorescence (see "Analyses for growth arrest" above) to determine the infection efficiency and confirm the growth arrest properties for each ARF mutant. Relative luciferase activities were calculated by normalizing luciferase readings to the percentage of cells expressing ARF or its mutants.

RESULTS

Comparison of the mouse and human ARF proteins revealed the greatest degree of homology between amino-terminal residues 1 to 14 and 21 to 25 (Fig. 1). This is consistent with earlier work showing that the first 62 amino acids (N62) of mouse ARF, which comprise its unique exon 1β, are sufficient and required for all of its functions (25, 46, 62). In those

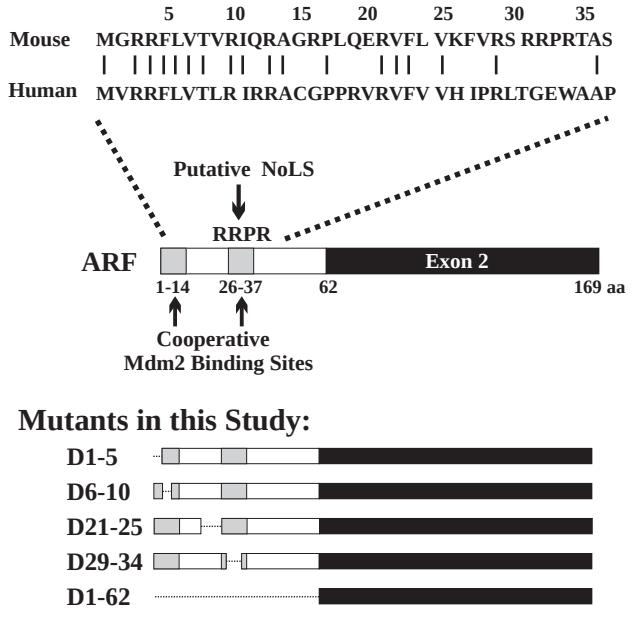

FIG. 1. Schematic representations of mouse ARF and its mutants. (Top) Alignment of mouse and human ARF protein sequences between amino-terminal residues 1 and 37. Identical amino acids are indicated by bars. (Middle) Structure of the mouse ARF protein, showing the positions of the two reported Mdm2 binding domains (shaded) and the predicted NoLS, RRPR. (Bottom) Structures of mouse ARF deletion mutants analyzed in this study. aa, amino acids.

studies, it was observed that the N62 mutant properly localized to nucleoli, bound and sequestered Mdm2 within nucleoli, and activated p53-dependent cell cycle arrest. In contrast, an ARF mutant lacking the first 62 residues, designated D1–62, was excluded from nucleoli and was devoid of antiproliferative activity (46, 62). To better define functional domains within ARF, we generated mutants with more discrete deletions in the amino terminus of murine ARF. This included mutants lacking residues 1 to 5, 6 to 10, 21 to 25, and 29 to 34. The last mutant, D29–34, eliminated an arginine-rich region, RSRRPR, that is common among nucleolar proteins and encompasses the putative nucleolar localization signal (62).

The growth-suppressive activity of ARF mutants was tested in *ARF*-null NIH 3T3 fibroblasts (Fig. 2). Cells were transduced with retroviruses derived from bicistronic plasmids encoding ARF or its mutants and the CD8 cell surface marker protein (46). At 2 or 3 days after infection, dual-color flow cytometry was performed to assess the DNA content of successfully infected CD8-positive cells that expressed vector, ARF, or its mutants. Figure 2A shows representative cell cycle distributions from a typical experiment, while data gathered from multiple experiments are displayed in Fig. 2B. Western blotting was performed in all experiments to confirm that similar levels of overexpressed ARF and its mutants were achieved (see Fig. 9A for ARF expression levels in the experiment in Fig. 2A). As expected, control cells expressing vector or the inactive mutant D1–62 continued to proliferate asynchronously whereas wild-type ARF induced a biphasic G_1 and G_2 phase growth arrest (26, 47, 55). Mutants lacking residues 1 to 5 (D1–5) and 29 to 34 (D29–34) actively inhibited growth, albeit slightly less effectively than did full-length ARF. In contrast, deletion mutants D6–10 and D21–25, like D1–62, were severely impaired in blocking cell cycle progression.

Similar results were obtained in assays testing the ability of the individual ARF mutants to inhibit DNA synthesis (Fig. 3). Infected cells were labeled with BrdU for 24 h or one full cell cycle, and its incorporation into replicating DNA within ARF-positive cells was determined by immunofluorescence. Approximately 95% of vector-infected cells incorporated BrdU under these conditions, whereas 90% of cells expressing full-length ARF were BrdU negative and growth arrested. Repeated analyses of D6–10 and D21–25 showed that they were functionally impaired in blocking DNA replication, although they generally exhibited more activity than did the fully compromised D1–62 mutant. By comparison, a statistically significant difference was seen between those mutants and D1–5, with the latter effi-

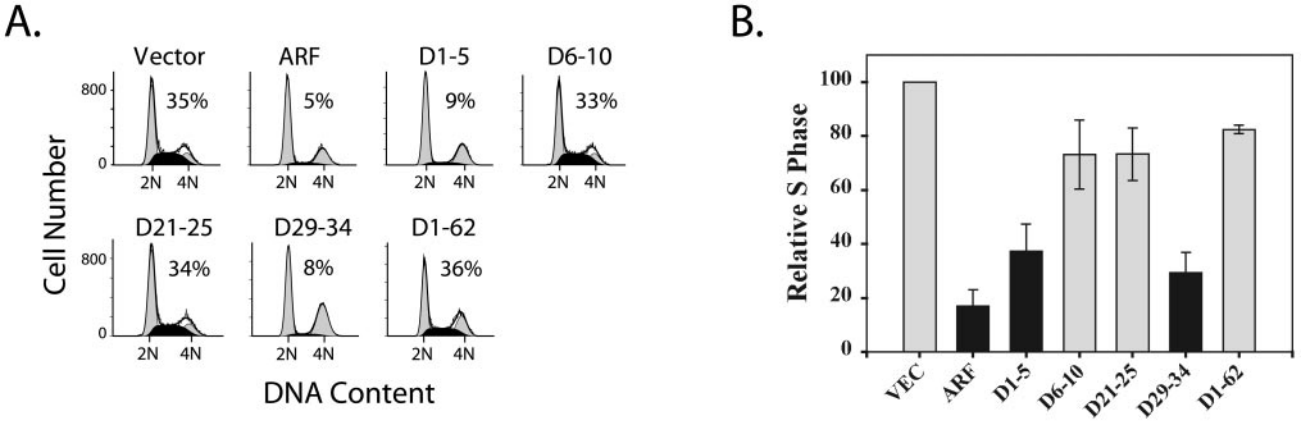

FIG. 2. Analysis of cell cycle distributions in fibroblasts expressing ARF mutants. *ARF*-null NIH 3T3-D1 cells were infected with bicistronic retroviruses encoding the cell surface protein, CD8 (vector), or CD8 plus wild-type mouse ARF or the different ARF deletion mutants, as indicated. The DNA content of successfully infected (CD8-positive) cells was analyzed 2 days after infection by dual-color flow cytometry. (A) Histograms from a representative experiment showing G_1 and G_2/M populations shaded in gray and S-phase cells highlighted in black. The percentage of cells in S phase is noted within each histogram. (B) The relative percent S phase for cells expressing ARF or its mutants was calculated relative to the vector control. Each value represents the mean and its standard deviation from at least three independent experiments.

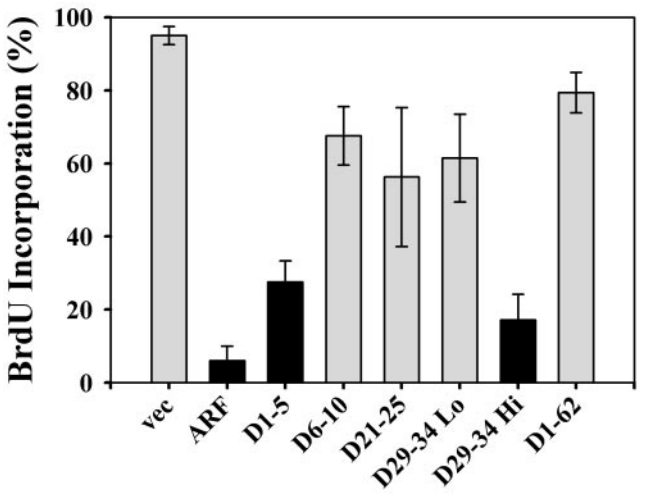

FIG. 3. Growth-suppressive activities of ARF mutants in BrdU incorporation assays. NIH 3T3 cells infected with retroviruses encoding empty vector, ARF, or ARF mutants, as indicated, were pulsed with BrdU for 24 h 1 day after infection. Cells were fixed and stained with antibodies to ARF and BrdU, and assayed by immunofluorescence on a confocal microscrope. The percentage of ARF-positive cells that were BrdU-positive was scored in three independent experiments (at least 100 cells per cell type counted per experiment). Error bars indicate standard deviations.

ciently inhibiting BrdU incorporation. These data were in agreement with those obtained by flow cytometry (Fig. 2), and indicated that residues 1 to 5 are largely dispensable for ARF function while amino acids 6 to 10 and 21 to 25 are required.

A recent study showed that an ARF mutant lacking residues 26 to 37 was excluded from the nucleolus in most cells (80% of cells examined) and was consequently inactive, presumably due to loss of the predicted nucleolar localization sequence, RRPR (61). Therefore, our finding that D29–34 was active (Fig. 2) was surprising, given that it also lacks the RRPR motif. BrdU incorporation assays revealed that in about 75% of cells, D29–34 was expressed at moderate to high levels within nucleoli or throughout the entire nucleus (D29–34Hi) and that in those cells the mutant was growth inhibitory (Fig. 3). However, in a minority of cells (25%), poor expression of D29–34 correlated with an inability to block DNA synthesis (D29–34Lo).

The subnuclear localization of ARF and its mutants was directly ascertained in infected NIH 3T3 cells by immunofluorescence using antibodies to ARF and fibrillarin, a marker protein specific to nucleoli (Fig. 4) (62). Nucleoli are sites of rDNA transcription and ribosome biogenesis that, in mammalian cells, vary in shape, size, and number within any given cell (14). Wild-type ARF, D1–5, D6–10, and D21–25 resided exclusively in nucleoli in all cells expressing the proteins, whereas D1–62 was always nucleoplasmic. These results were expected, given that larger deletion mutants of ARF lacking residues 1 to 14 or 15 to 25 are also nucleolar (61). Most cells expressing D29–34 exhibited punctate nucleolar staining or contained the protein throughout the entire nucleus, including the nucleoli (D29–34Hi). Careful examination of cells poorly expressing D29–34 revealed low levels of protein in the nucleoplasm as well as in nucleoli (D29-34Lo; note the yellow color in the merged image showing colocalization of D29–34 with fibrillarin). Analyses of 0.3-μm optical sections on a confocal micro-

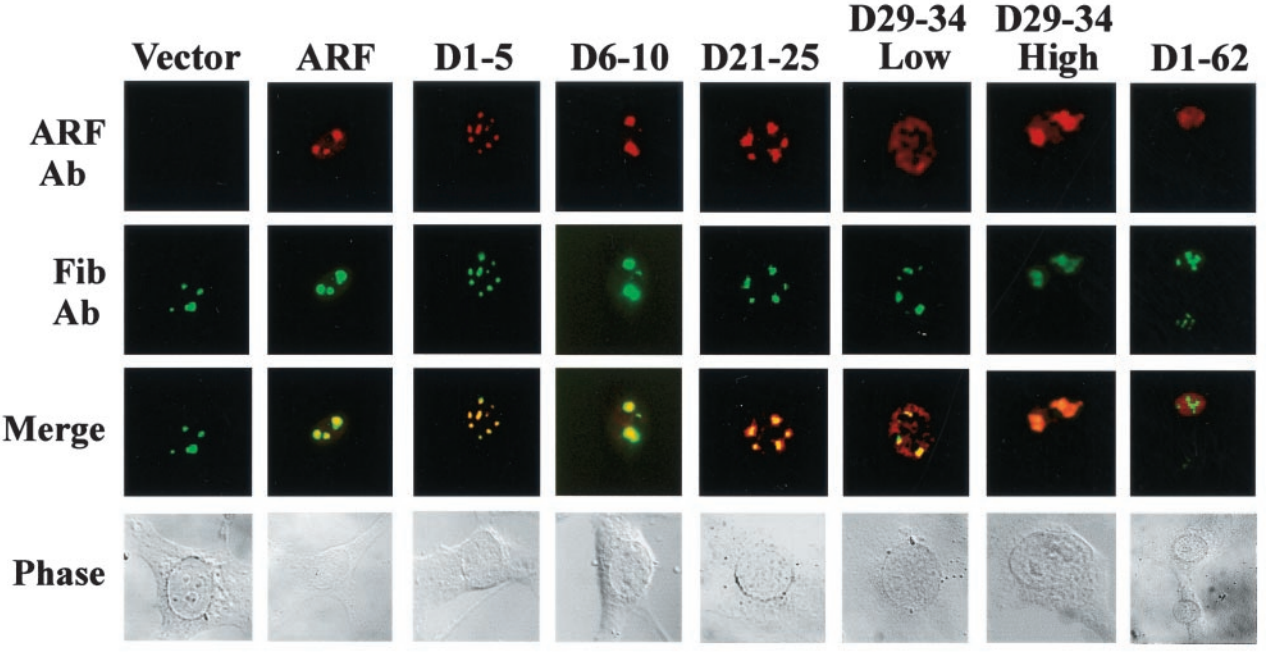

FIG. 4. Subnuclear localization of ARF mutants. *ARF*-null NIH 3T3 cells infected with vector, ARF, or the indicated ARF mutant retroviruses were examined by immunofluorescence after staining with antibodies (Ab) to ARF (red, top row) and the nucleolar marker protein fibrillarin (Fib) (green, second row). Colocalization between ARF and fibrillarin was revealed in the merged images (yellow, third row). Individual cells were visualized by phase-contrast microscopy (bottom row). The localization pattern of D29–34 in cells expressing moderate to high levels of the protein (D29–34 High) is compared with that in cells expressing low levels (D29–34 Low). Although not shown, the localization of the D29–34 mutant was rigorously examined in horizontal optical slices of 0.3 μm obtained by confocal microscopy.

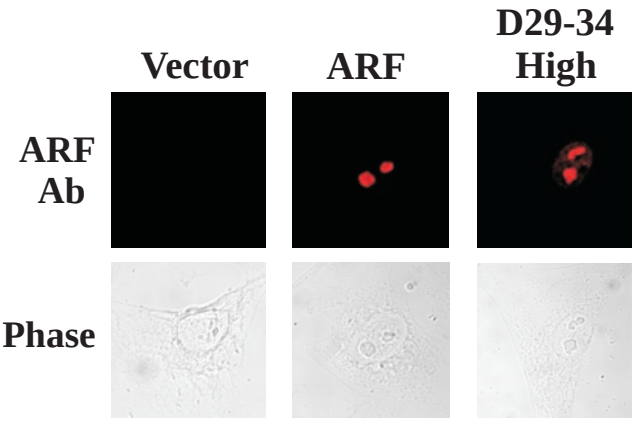

FIG. 5. Localization of ARF or its mutants is not altered in the absence of Mdm2. *ARF/p53/Mdm2*-null MEFs were infected with retroviruses encoding vector, ARF, or the deletion mutant D29–34. Cells were examined by immunofluorescence after staining with antibodies (Ab) to ARF (red, top row). Nucleoli within individual cells are visible in the phase-contrast images (bottom row). A representative picture of cells expressing moderate to high levels of D29–34 (70% of cells) is shown.

scrope confirmed the presence of D29–34 in nucleoli in most cells, although some low expressors exhibited complete exclusion of the mutant from nucleoli (data not shown), as reported previously for D26–37 (61). These findings showed that the putative NoLS between residues 29 and 34 of ARF is not required for nucleolar localization and suggested that its loss may impair nucleolar retention of ARF.

We tested the possibility that Mdm2 contributed to the mobilization of these ARF mutants to nucleoli or, conversely, partially retained D29–34 in the nucleoplasm. Previous studies showed that Mdm2 contains a cryptic nucleolar localization signal that is unmasked by binding to ARF, and in cells ectopically expressing both proteins, it is required for localization of the complex to the nucleolus (36, 61). We observed identical patterns of localization for all mutants in cells lacking Mdm2. Specifically, wild-type ARF and the nucleolar mutants (D1–5, D6–10, and D21–25) remained exclusively nucleolar in MEFs lacking p53, ARF, and Mdm2 (Fig. 5 and data not shown). Moreover, as in Mdm2-positive cells, D29–34 was nucleolar at higher expression levels (in approximately 75% of cells) (Fig. 5) and primarily nucleoplasmic when expressed at relatively low levels (in 25% of cells [data not shown]). These data indicate that the subnuclear distributions of ARF mutants are not Mdm2 dependent, consistent with earlier work showing that wild-type ARF localization is not dependent on Mdm2 (60, 62).

Two reported requirements for ARF function are that it must be nucleolar and that it must bind and import Mdm2 into that compartment (61, 62). Since ARF mutants D6–10 and D21–25 localized properly to nucleoli yet lacked growth-suppressive activity, we tested if they were inactive due to an inability to bind and import Mdm2. Mobilization of Mdm2 into the nucleolus by ARF depends on a direct interaction between the two proteins (35, 36, 62), so we first examined the ability of each mutant to associate with Mdm2 when both proteins were efficiently expressed in human *ARF*-null U20S cells (Fig. 6). Western blots of proteins precipitated with antibodies to either ARF or Mdm2 showed that both active (wild-type, D1–5, and D29–34) and inactive (D6–10, D21–25, and D1–14) forms of ARF interacted with Mdm2 in vivo. Interestingly, the ability of ARF antibodies to precipitate the complex was consistently diminished for mutants D1–5, D6–10, D21–25, and D1–14 compared to wild-type ARF or D29–34. This could reflect altered conformation of ARF mutants in the complex, perhaps due to reduced Mdm2 binding affinity. As controls in this assay, two ARF mutants previously shown to lack Mdm2 binding ability (D1–62 and D1–14;D26–37) failed to associate with Mdm2 (61, 62, 64). Overall, these data were consistent with an earlier finding that both Mdm2 interaction domains (residues 1 to 14 and 26 to 37) must be simultaneously deleted to eradicate Mdm2 binding (61).

The ability of ARF mutants to import Mdm2 into nucleoli was assayed by immunofluorescence in NIH 3T3 fibroblasts. Since we were unable to detect the extremely low levels of endogenous Mdm2 in NIH 3T3 cells by immunofluorescence, even in those expressing wild-type ARF, we examined cells that were cotransfected with ARF and Mdm2 constructs. This approach is commonly used in ARF-Mdm2 interaction and localization studies (35, 36, 61, 62), and it was used to demonstrate that the inactive D1–14 ARF mutant resided in nucleoli but failed to mobilize Mdm2 out of the nucleoplasm (61). As shown in Fig. 7, wild-type ARF and its mutants D1–5, D6–10, D21–25, and D29–34 induced complete relocalization of Mdm2 into nucleoli. Unlike the earlier study (61), the expression of D1–14 in nucleoli always coincided with import of Mdm2. By comparison, expected results were obtained in control cells expressing exogenous Mdm2 alone or Mdm2 plus the nucleoplasmic ARF mutant, D1–62. In both cases, Mdm2 was localized in the nucleoplasm. Identical results were observed in both NIH 3T3 and COS cells transfected with different

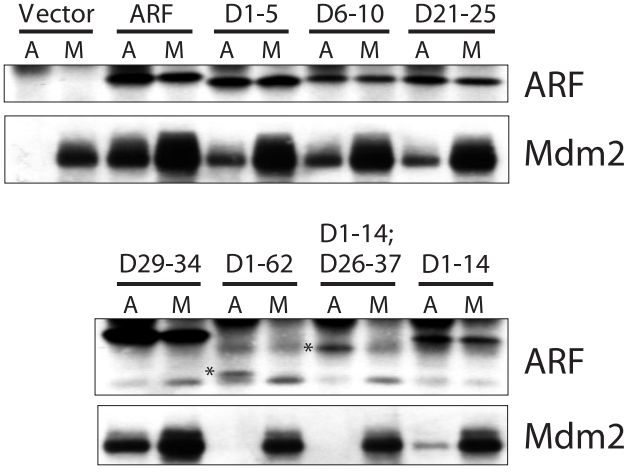

FIG. 6. Inactive ARF deletion mutants, D6–10 and D21–25, bind to Mdm2 in vivo. U2OS cells were transfected with expression constructs encoding wild-type Mdm2 plus empty vector, wild-type ARF, or the indicated ARF mutants. Complexes between ARF and Mdm2 were identified by immunoprecipitation with antibodies to Mdm2 (M) and ARF (A), followed by Western blotting with both antibodies, as indicated to the right of each blot. The inactive ARF mutants which lack Mdm2 binding capability (D1–62 and D1–14;D26–37) are highlighted by asterisks.

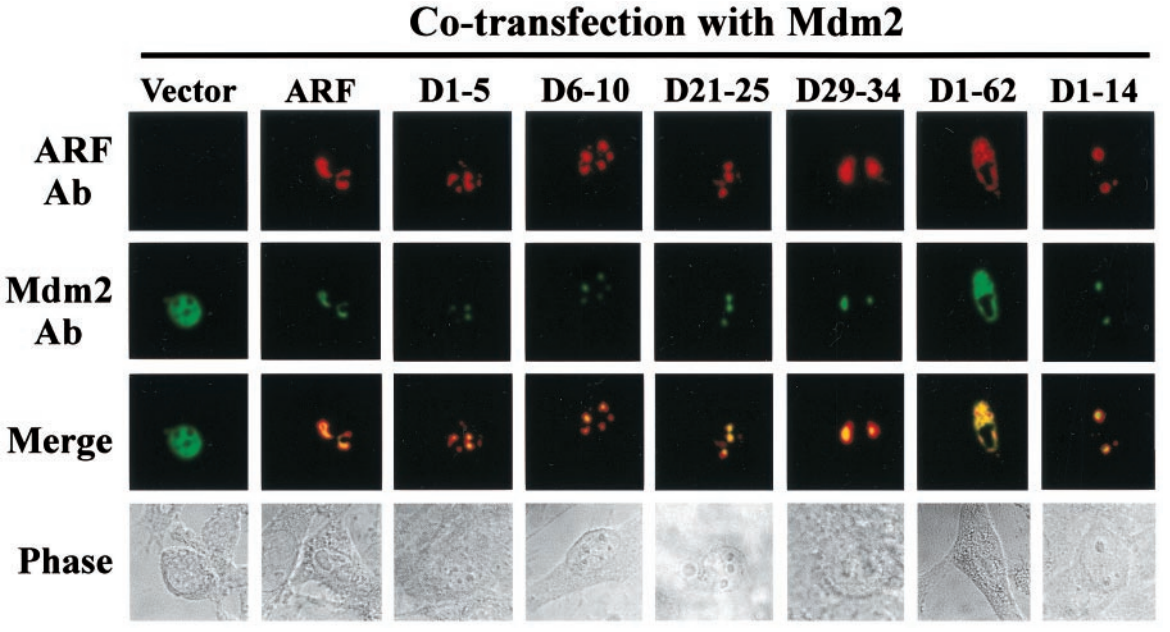

FIG. 7. Inactive ARF deletion mutants, D6–10 and D21–25, import exogenous Mdm2 into nucleoli. NIH 3T3 cells were transfected with an expression vector encoding Mdm2 plus empty vector, ARF, or the indicated ARF mutants. The localization of ARF (red, top row) and Mdm2 (green, second row) was assayed by antibody (Ab) staining and confocal microscopy. Colocalization between ARF and Mdm2 was observed (merged images, third row) in the nucleoli of all cells for all mutants tested, except D1–62, which remained nucleoplasmic with Mdm2. Nucleolar localization was confirmed in parallel studies examining ARF with fibrillarin (data not shown).

amounts of ARF plasmids (0.35 to 1 μg) plus equal quantities of either mouse or human Mdm2 constructs (data not shown). Since gross overexpression of Mdm2 and ARF can lead to their accumulation in cellular aggregates which are not nucleoli, termed nuclear bodies (62, 65), we confirmed that ARF and Mdm2 were nucleolar by simultaneously staining cells with antibodies to ARF and fibrillarin. ARF and its mutants, except D1–62, colocalized with fibrillarin in all cells (data not shown), providing correlative evidence that Mdm2 must also be nucleolar. These results suggested that the inability of D6–10 and D21–25 to suppress growth was not a consequence of mislocalization or an inability to bind and import Mdm2.

To better assess the effects of ARF mutants on Mdm2 import, we assayed the localization of endogenous human Mdm2 (Hdm2) in U2OS cells transfected with each ARF construct. In *ARF*-null U2OS cells expressing empty vector alone, relatively high basal levels of endogenous Hdm2 were detected throughout the nucleus, with some evidence of nucleolar exclusion (Fig. 8). Expression of wild-type ARF caused the upregulation of Hdm2, but, surprisingly, Hdm2 relocalization to nucleoli did not occur in all ARF-positive cells. Rather, we observed that Hdm2 import coincided with high levels of ARF expression while cells expressing lower levels of ARF generally failed to relocalize Hdm2 (Fig. 8B). Likewise, the population of cells robustly expressing the nucleolar ARF mutants (D1–5, D6–10, D21–25, D29–34, and D1–14) sequestered endogenous Hdm2 in nucleoli (Fig. 8A). Among those mutants, the efficiency of Hdm2 redistribution was comparable for both growth-inhibitory and active forms of ARF. D1–62 was used as a negative control for Hdm2 import since it normally localizes to the nucleoplasm. However, in 7% of cells, D1–62 was expressed at unusually high levels throughout the nucleoplasm and nucleoli, and this correlated with its ability to import Hdm2. These results suggested that excessive levels of ARF expression achieved in transfected U2OS cells could drive Hdm2 relocalization.

We had previously noticed that a U2OS derivative cell line, NARF, expresses relatively low levels of inducible human ARF in response to IPTG (55). Therefore, we examined the localization of endogenous Hdm2 in NARF cells treated with IPTG for 48 h. Those cells undergo complete growth arrest after 2 days of IPTG treatment (34, 55). However, as recently reported by Llanos et al. (34), Hdm2 remained in the nucleoplasm in all IPTG-treated NARF cells expressing ARF (Fig. 8). This result clearly indicated that Mdm2 import is not required for ARF-induced growth arrest. It also suggested that the ability of various ARF mutants to import Mdm2 may not be informative with respect to characterizing their growth-suppressive activities, in keeping with our inability to distinguish between active and inactive ARF mutants based on Mdm2 import capabilities.

Growth suppression by ARF is associated with the accumulation of p53 and its target gene, Mdm2 (45, 54, 55, 64). To better characterize the functional effects of the different ARF mutants, changes in the expression of these proteins in response to ARF or its mutants were examined by immunoblotting of whole-cell lysates from infected cells (Fig. 9A). All ARF proteins were expressed at high and essentially equivalent levels, and no endogenous ARF was detected in vector-infected, *ARF*-null NIH 3T3 cells (Fig. 9A, top panel) (47). Introduction of wild-type ARF stimulated the marked accumulation of p53 and Mdm2 compared to growing, vector-infected cells (middle panels). Another transcriptional target of p53, cyclin G1, was also found to accumulate in response to ARF (bottom panel).

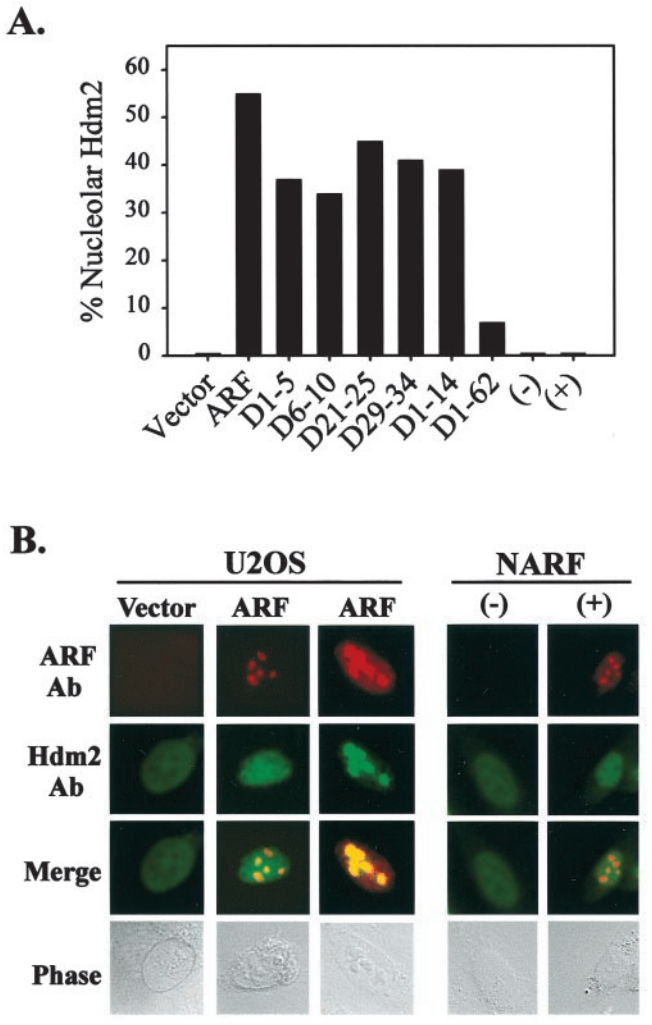

FIG. 8. Lack of correlation between ARF activity and Hdm2 import. Human U2OS cells were transfected with empty vector, full-length ARF, or ARF mutant plasmids. Hdm2 and ARF localization were determined by immunofluorescence 2 days later. (A) Graphical representation of the efficiency of Hdm2 nucleolar import by different forms of ARF (data averaged from two or more experiments). (B) Localization of exogenous wild-type ARF (red, top row) and endogenous Hdm2 (green, second row), either in U2OS cells transfected with vector or ARF plasmids or in NARF cells that were treated with (+) IPTG for 2 days or left untreated (−). Although not shown, IPTG treatment caused complete G_1 and G_2 phase growth arrest of NARF cells, as reported previously (34, 55). Ab, antibody.

These results were expected and indicative of p53 activation by ARF. Surprisingly, none of the ARF mutants induced the accumulation of p53, even the active mutants D1–5 and D29–34. Likewise, Mdm2 and cyclin G1 were only minimally increased by D1–5 and D29–34 compared to vector controls or cells expressing the inactive mutants D6–10, D21–25, and D1–62. Another transcriptional target of p53, the Cdk inhibitor p21, was not upregulated by any of the mutants tested (data not shown).

The lack of p53 stabilization in cells expressing D1–5 and D29–34 implied a p53-independent mechanism of action for those mutants. However, the G_1 and G_2 phase growth arrest phenotype of cells expressing those mutants mirrored that of a p53-dependent arrest (26, 60). To test whether p53 was activated by D1–5 and D29–34 in the absence of p53 stabilization, p53 reporter assays were performed (Fig. 9B). NIH 3T3 cells stably expressing a p53 luciferase reporter construct were infected with empty vector, ARF, or ARF mutant retroviruses, and 2 days later luciferase assays were performed. Both wild-type ARF and D1–5 activated p53 transcription five- to nine-

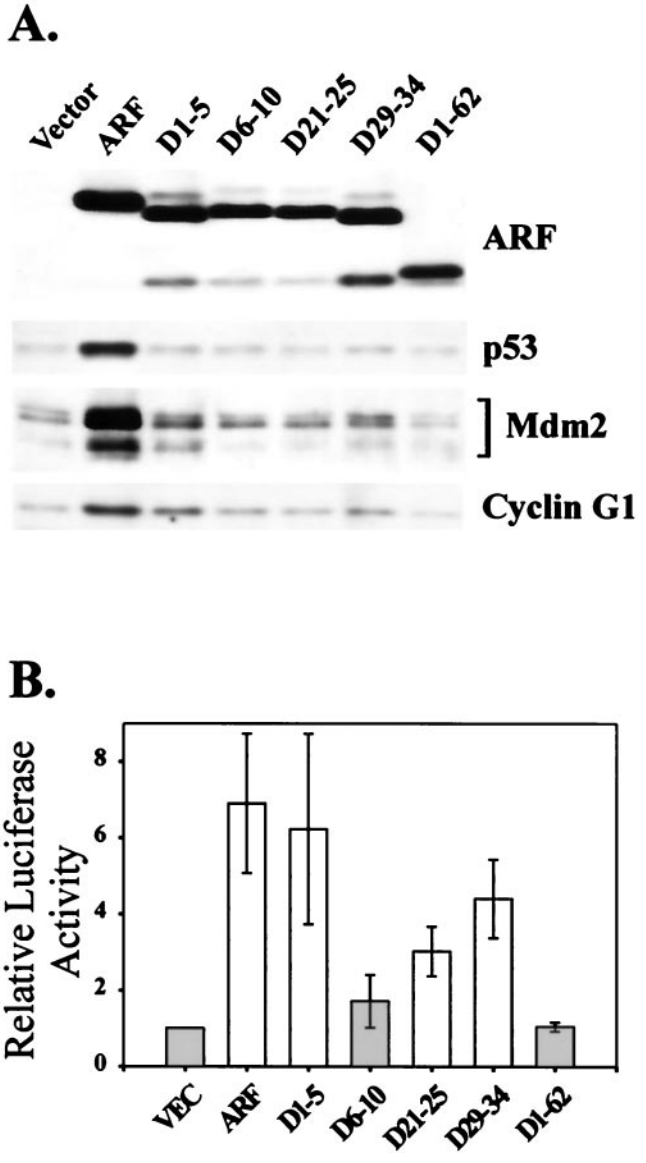

FIG. 9. Growth-inhibitory ARF mutants, D1–5 and D29–34, activate but do not stabilize p53. (A) NIH 3T3 cells infected with the indicated retroviruses were harvested 2 days after infection and lysed, and equivalent amounts of total cellular protein (50 μg per lane) were analyzed by Western blotting. The expression of ARF, p53, and two p53 transcriptional targets, Mdm2 and cyclin G1, was examined. (B) NIH 3T3 cells stably expressing a p53 luciferase reporter construct were infected with the indicated retroviruses, and luciferase assays were performed 2 days after infection. Relative p53-dependent luciferase activities were determined by normalizing luciferase readings to the percentage of cells expressing ARF or its mutants (determined by immunofluorescence). Standard deviations are shown for three independent experiments.

TABLE 1. Growth arrest ability of mouse ARF mutants in MEFs lacking ARF, Mdm2, and p53[a]

Retrovirus	% of BrdU-positive cells
Vector	92 ± 5
ARF	42 ± 9
D1–5	43 ± 4
D6–10	78 ± 5
D21–25	87 ± 5
D29–34Lo	85 ± 15[b]
D29–34Hi	30 ± 0[b]
D1–14	80 ± 1[b]

[a] At 3 days after infection with the indicated retroviruses, cells were BrdU labeled for 24 h and then stained with ARF and BrdU antibodies for immunofluorescence. ARF-positive cells were counted for BrdU positivity in three experiments. Each value represents the mean and its standard deviation.
[b] Data were averaged from two experiments.

fold above the background levels observed in vector-infected cells. A less robust but nonetheless significant induction of p53-dependent luciferase activity was exerted by D29–34. Interestingly, D21–25 also partially activated p53. By comparison, the inactive mutants D6–10 and D1–62 had essentially no effect on the basal levels of p53 activity. The findings for D1–5 and D29–34 suggest that ARF has the ability to activate p53 in the absence of detectable p53 stabilization.

Since ARF was recently shown to have p53- and Mdm2-independent function (60), we tested whether our active mutants could suppress growth in the absence of both proteins. ARF and its mutants were expressed by infection in MEFs lacking p53, Mdm2, and ARF, and their ability to suppress growth was measured in BrdU incorporation assays. As shown in Table 1, D1–5 and D29–34 behaved like wild-type ARF in blocking DNA synthesis whereas the mutants found to lack activity in NIH 3T3 fibroblasts (D6–10, D21–25, and D1–14) were also unable to inhibit growth in this genetic background. These results strengthen the idea that residues 1 to 5 and 29 to 34 are dispensable for ARF activity regardless of the p53 status within the cells. In contrast, residues 6 to 10 and 21 to 25 are required for both p53-dependent and p53-independent function of ARF.

DISCUSSION

ARF can suppress growth through multiple pathways that are either p53 dependent or p53 independent. In p53-positive cells, the current model is that ARF sequesters Mdm2 in nucleoli, thereby stabilizing p53 and activating p53-dependent G_1 and G_2 phase cell cycle arrest (54). In cells lacking p53 and Mdm2, ARF suppresses growth through undefined mechanisms (60). This study narrows the critical functional domains required for ARF-induced growth arrest, yet it also provides additional complexity to our understanding of ARF signaling. We showed that amino-terminal residues 6 to 10 and 21 to 25 of mouse ARF are essential for growth inhibition by ARF in both p53-positive and p53-negative cells. In contrast, residues 1 to 5 and 29 to 34 are largely dispensable for ARF activity. We also demonstrated that the growth-suppressive activity of ARF does not correlate with ability to relocalize Mdm2 and that ARF can activate p53 and inhibit growth without stabilizing p53. These observations strongly suggest that additional factors or events other than Mdm2 import are critical for ARF-mediated growth arrest.

Deletion of highly conserved residues 6 to 10 and 21 to 25 in mouse ARF showed that both domains are required for the growth-inhibitory activity of ARF. The inactivity of D21–25 was somewhat unexpected because an ARF mutant lacking residues 15 to 25 retains growth-inhibitory activity (61). It is possible that deletion of residues 21 to 25 induces inactivating conformational changes in ARF. However, we found that an alanine substitution mutant (21–25Ala) also lacks growth-inhibitory activity (67 and 80% BrdU incorporation in assays identical to those in Fig. 3 [data not shown]). These results indicate an important role for residues 21 to 25 in ARF function when residues 15 to 20 remain intact, but no role when the entire region is deleted.

The most likely explanation for the inactivity of D6–10 and D21–25 is that those mutants are impaired in Mdm2 binding. Each deleted region contains an RFLV amino acid motif that was recently proposed to mediate Mdm2 binding, based on data from peptide binding studies (39) and the solution structure of a mouse ARF fragment encompassing residues 1 to 37 (7). The first RFLV motif (located at residues 4 through 7) appears to mediate a higher-affinity association with Mdm2 (39, 61), which may correlate with observations that D6–10 was more severely compromised than D21–25 in activating p53 in reporter assays. Notably, we found that both mutants properly localized to nucleoli and associated with Mdm2 in vivo. This suggests that the single Mdm2 binding region present in each mutant is sufficient to mediate an interaction, consistent with earlier findings (61). Still, the association between Mdm2 with D6–10 or D21–25 would presumably be of lower affinity. Our binding data suggest that this might be the case since complexes between Mdm2 and the mutant proteins were ineffectively precipitated by ARF antibodies, hinting at conformational differences in ARF mutants due to altered Mdm2 binding characteristics. However, a potentially weakened Mdm2-ARF interaction did not reduce the ability of those mutants to import exogenous Mdm2, and endogenous Hdm2 was imported at equal efficiency by both inactive and active nucleolar forms of ARF. At face value, such results suggest that nucleolar localization and Mdm2 import are not sufficient for ARF function. Moreover, they imply that other factors associate with the ARF amino terminus which are required for its growth-suppressive activity.

Recent findings indicate the existence of other ARF binding proteins. First, ARF can inhibit growth in cells lacking p53 and Mdm2 (60), revealing that other factors besides p53 and Mdm2 can execute ARF's functions. Second, ARF associates with certain members of the E2F transcription factor family and may target them for degradation (12, 38). Third, ARF can form complexes with spinophilin, a regulatory subunit of the protein phosphatase 1 catalytic subunit (59), as well as topoisomerase I (27), MdmX (24), and a cytoplasmic peroxisomal protein, Pex19p/HK33/PXF (56). Finally, we recently identified a novel nucleolar protein that associates with active forms of ARF but fails to interact with inactive ARF mutants D21–25 and D1–14;D26–37 (X. Luo and D. Quelle, unpublished results). While the significance of the interactions between ARF and the aforementioned proteins in ARF-mediated tumor suppression remains to be established, such data are consistent

with the hypothesis that alternative ARF binding proteins are important for ARF function.

The likelihood that additional factors besides Mdm2 play a key role in ARF pathways is bolstered by findings that ARF can inhibit growth without relocalizing endogenous Mdm2 out of the nucleoplasm. This was observed in an ARF-inducible U2OS cell line (NARF) and in primary human fibroblasts in which endogenous ARF was activated by E2F (34). Others demonstrated that oncogenic *ras* activates ARF-induced growth arrest in murine keratinocytes without relocalization of Mdm2 to nucleoli (32). In this study, we obtained the same results as Llanos et al. using the NARF cell system (34). A conceptual dilemma consequently arises because the above observations contradict the existing model in which ARF functions by sequestering Mdm2 in nucleoli. The model is supported by earlier studies, as well as by some data presented here, showing that ARF imports exogenous or endogenous Mdm2 (36, 61, 62). How can the two bodies of evidence be reconciled? One possibility is that ARF expression levels are a key determinant of Mdm2 import. Our experiments showed that endogenous Hdm2 is selectively relocalized in transfected U2OS cells which express extremely high levels of ARF whereas Hdm2 remained nucleoplasmic in U2OS and NARF cells expressing low levels of ARF. Therefore, we suggest that overexpression studies assaying Mdm2 import must be interpreted with caution, even though they are typically used to assess ARF-Mdm2 interactions (35, 36, 61, 62). Importantly, ARF overexpression does not seem to interfere with assessment of its other activities, such as growth arrest, localization, or p53 stabilization and activity, since we can distinguish between inactive and active mutants in those assays.

Does this mean that Mdm2 relocalization is not a biologically relevant component of ARF-induced growth suppression? It is clear that Mdm2 import is not required for ARF function in response to E2F or Ras (32, 34). However, earlier work showed that endogenous Mdm2 accumulated in nucleoli with ARF in MEFs overexpressing Myc or undergoing cellular senescence (62). Thus, the stimulus which activates ARF, and possibly the cellular context, may contribute to whether Mdm2 is mobilized into nucleoli. Even if that is true, the problem of how to interpret data from import studies involving overexpression of ARF or Mdm2 mutants still exists. In this study, we were unable to correlate ARF function with Mdm2 relocalization since both active and inactive nucleolar forms of ARF imported Mdm2. We considered the possibility that our assay was flawed since we could not reproduce work showing that the inactive mutant D1–14 fails to sequester Mdm2 in nucleoli (61), but we concluded that this is unlikely for several reasons. First, the only technical difference between the two studies was minor and involved the use of an epitope-tagged Hdm2 construct by Weber et al. (61). Second, changes in expression levels of Mdm2 with ARF, D1–14, or other nucleolar mutants did not alter their ability to colocalize. Instead, we found that comparably low levels of ARF (wild type or mutant) were relocalized to the nucleoplasm by high levels of Mdm2 (data not shown), further supporting an interaction between the two proteins. Since both studies were well controlled, at present we cannot explain the reason for the different results. Ultimately, measurement of the actual Mdm2 binding affinities for each ARF mutant will be informative in determining whether Mdm2 binding correlates with their growth-suppressive activities.

In circumstances where ARF does not cause quantitative relocalization of Mdm2 (Fig. 8) (32, 34), retention of even a small number of Mdm2 molecules in nucleoli by ARF may serve to increase the level of free p53 enough to allow its stabilization and activation. Alternatively, it has been suggested that a small number of ARF molecules in the nucleoplasm may be sufficient to block Mdm2 activity, stabilize p53, and cause growth arrest (32, 34, 39). Consistent with that notion is the ability of nucleoplasmic forms of ARF (when overexpressed as green fluorescent protein (GFP) fusions) to stabilize and activate p53 (34, 39). The latter idea is attractive because it is not understood how Mdm2 is relocalized to the nucleolus or why p53 remains in the nucleoplasm when it can associate with both Mdm2 and ARF (25, 34, 55, 64). On the other hand, why is ARF located in the nucleolus, if not to carry out its function? Indeed, poor expression of D29–34 in nucleoli and localization of other ARF mutants in the nucleoplasm (D1–62, D26–37, and D1–14;D26–37) coincides with their inability to block growth (Fig. 3 and 4) (61, 62). Unfortunately, it is difficult to separate the contributions of nucleoplasmic localization versus loss of Mdm2 binding to the inactivity of such mutants.

Several studies identified arginine-rich nucleolar localization signals (NoLS) within mouse and human ARF proteins (34, 35, 48, 61, 62, 65). We directly tested the importance of the predicted NoLS (^{31}RRPR^{34}) for proper mouse ARF localization and found that its loss had minimal effects. Most cells expressed the mutant in nucleoli or throughout the entire nucleus, and only extremely poor expression disrupted its ability to localize to nucleoli. We also found that residues 26 to 37 encompassing the RRPR domain were not sufficient for nucleolar targeting (data not shown). Fusion of those residues, or amino acids 1 to 14 or both 1 to 14 and 26 to 37, to GFP could not direct GFP to nucleoli, similar to results obtained in studies of human ARF peptides (34). Thus, our results indicate that the ^{31}RRPR^{34} sequence is neither required nor sufficient for nucleolar localization. Another group likewise showed that deletion of residues 26 to 37 did not abolish nucleolar targeting since 20% of cells expressed the mutant in nucleoli (61). However, since the majority of cells exhibited nucleoplasmic mislocalization and inactivity of D26–37, it is conceivable that the additional residues lost in D26–37 compared to D29–34 directly contribute to nucleolar localization, and/or contribute to the proper conformation of ARF required for its activity.

In this study, ARF mutants lacking residues 1 to 5 or 29 to 34 retained growth-suppressive activity yet failed to stabilize p53. Despite the lack of p53 accumulation, two observations suggest that both mutants mediated a p53-dependent growth arrest. The primary reason is that D1–5 and, to a lesser extent, D29–34 effectively induced p53 transcriptional activity in reporter assays. Also, NIH 3T3 fibroblasts ($p53^{+/+}$) expressing D1–5 and D29–34 for 2 days were efficiently arrested in the G_1 and G_2 phases. That phenotype of growth arrest is identical to that elicited by wild-type ARF in p53-positive cells (26, 47, 55) and quite unlike the delayed G_1 phase block exerted by ARF and these mutants in *p53*/*ARF*/*Mdm2*-null fibroblasts (Table 1) (60). Notably, both mutants induced a marginal upregulation of Mdm2 and cyclin G1 proteins and no upregulation of p21,

despite apparent p53 activation. This may reflect activation of a smaller pool of p53 molecules, altered specificity of p53 transactivation, and/or altered posttranscriptional mechanisms governing the stability of Mdm2 and cyclin G1. Regardless, the level of p53 activation achieved by D1–5 and D29–34 appears sufficient for growth arrest. Interestingly, the moderate threefold activation of p53 mediated by D21–25 is not significantly lower than that mediated by D29–34. Since D21–25 has little growth-inhibitory activity, this result suggests either that there is a certain threshold of p53 activity required for ARF-induced growth arrest or that other events besides p53 activation are involved.

Activation of p53 by ARF without detectable p53 stabilization implies ARF-mediated posttranslational modification of p53. It is known that p53 is activated by phosphorylation and acetylation (15), and others have shown that p53-mediated transcription can be upregulated in the absence of any increase in p53 protein levels (22, 63). Moreover, p53 accumulation alone is insufficient to induce its transcriptional activities (3). Although ARF does not activate p53 via phosphorylation on serine 15 of p53 (6), its overexpression has recently been reported to induce p53 acetylation (23). Given that Mdm2 can inhibit p300/CBP-mediated acetylation of p53 (23), that p300 interacts with Mdm2 in a region overlapping the ARF binding site (16, 39, 61), and that ARF can abrogate Mdm2-mediated suppression of p53 acetylation (23), it is possible that binding of ARF to Mdm2 competitively releases p300 and allows it to activate p53. Such a model was originally proposed by Midgley et al. (39), and it provides a logical explanation for how active ARF mutants that fail to stabilize p53 can still activate p53 transcription.

What the model discussed above does not explain is why D1–5 and D29–34 fail to stabilize p53. It is conceivable that the binding of either mutant to Mdm2 causes the displacement of p300 (and consequent activation of p53) but is not sufficient to alter Mdm2 conformation and impair its ubiquitin ligase activity. This would result in low levels of p53 due to continued Mdm2-mediated degradation, but existing p53 molecules would be activated due to acetylation. In support of that notion, D1–5 lacks half of the predicted high-affinity Mdm2 binding site, ^{4}RFLV^{7}, and it appears to bind with reduced affinity to Mdm2 in mammalian cells. If this hypothesis is true, then ARF function in p53-positive cells does not strictly depend on inhibition of Mdm2 ubiquitin ligase activity.

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