The tumor suppressor protein ARF (alternate reading frame) inhibits MDM2 to stabilize and activate the functions of p53. Here we provide evidence for an additional activity of ARF that attenuates cell cycle progression independently of p53 activation. We show that ARF interacts with c-Myc independently of MDM2 or p53. Consequently, ARF relocates c-Myc from the nucleoplasm to the nucleolus. Binding and relocalization by ARF correlate with an inhibition of the c-Myc-activated transcription in both p53-positive and -negative cells. Using inducible cell lines, we show that the wild type ARF, but not a mutant, inhibits expression of the c-Myc-induced genes before inhibiting S phase. Moreover, ARF inhibits Mdc2-induced progression into S phase in cells lacking p53 or expressing a defective p53, indicating that ARF inhibits the S phase stimulatory function of c-Myc independently of p53. Our results strongly suggest that c-Myc is a bona fide target of ARF and that ARF attenuates c-Myc independently of the ARF-p53 axis.

The alternate reading frame (ARF)/INK4A locus encodes two tumor suppressor proteins: p16Ink4a and a 19-kDa ARF protein in mice or 14-kDa ARF protein in humans (1–3). This locus is disrupted in a large number of tumors (4). The frequent inactivation of ARF/INK4A is a fail-safe mechanism that protects cells against the tumorigenic consequences of oncogene activation (3). The ARF gene has been linked to p53. It has been shown that ARF can stabilize and stimulate the activities of p53 (9–17), and it does so by regulating MDM2, which is an inhibitor of p53. MDM2 has been shown to possess a p53-specific E3 ubiquitin ligase activity (18–21). It binds to p53 and causes ubiquitination followed by proteolysis of p53. ARF associates with MDM2 and inhibits its ability to ubiquitinate p53 (22). Recent studies suggested additional p53-independent functions of ARF. For example, it has been shown that the sequences of ARF involved in MDM2 regulation and p53 stabilization are not sufficient for the growth suppression function of ARF (23). Evidence for a p53-independent function of ARF came from studies that compared tumor frequencies in mice lacking ARF, MDM2, and p53 with mice lacking p53 and MDM2 or p53 alone (24). Mice nullizygous for ARF, p53, and MDM2 developed tumors at a frequency greater than those observed in mice lacking both p53 and MDM2 or p53 alone (24). Moreover, reintroduction of ARF in fibroblasts lacking ARF, p53, and MDM2 caused a G1 arrest (24), suggesting that ARF can interact with targets other than p53 and MDM2 to inhibit cell proliferation.

Recent studies provided evidence that ARF could associate with certain members of the E2F family of transcription factors (25–27) and induce their degradation through the ubiquitin-proteasome pathway (26, 27). The degradation is associated with a relocalization. For example, ARF expression caused relocalization of E2F1 from mainly nucleoplasmic to mainly nucleolar. The E2F family transcription factors are involved in cell proliferation and are targets of a variety of oncogenic pathways. However, the significance of the ARF-induced proteolysis of the E2F family of factors with regard to the tumor suppression function of ARF has yet to be established. Recent studies also have linked the antiproliferative functions of ARF to ribosome biogenesis (28). It has been shown that p19ARF inhibits the production of ribosomal RNA, especially the processing of 47/45 and 32 S rRNA to mature 18 S and 28 S rRNA. This function of ARF is independent of both p53 and Mdm2 but depends upon the highly conserved first 14 amino acids at the N terminus of the ARF protein (28).

The c-Myc protein is a bHLHZip transcription factor that is involved in cell proliferation, differentiation, and apoptosis (29, 30). The gene encoding c-Myc is also deregulated in many
Myc-ARF Interaction Inhibits the Functions of Myc

Myc, in conjunction with the heterodimeric partner Max, stimulates expression of genes through the recognition of E box sequence elements. A number of target genes have been identified that show a dependence on Myc for their expression in vivo (33, 34). Myc proliferated faster when taken from ARF–/– background compared with the MEFs from ARF+/+ background (45), suggesting that Myc can directly or indirectly regulate the function of Myc. Here we show that ARF associates with c-Myc and relocalizes c-Myc from the nucleoplasm to the nucleolus. Moreover, ARF inhibits c-Myc-activated transcription. Also, using an inducible system for ARF expression, we show that ARF inhibits expression of the c-Myc-induced genes prior to G1 arrest. Moreover, we show that ARF inhibits the function of c-Myc in a p53-independent manner. Results are consistent with a role of c-Myc-inhibition in the G1 arrest of cells treated with ARF.

MATERIALS AND METHODS

Expression Plasmids—The expression plasmid expressing hemagglutinin-tagged p19ARF14–14 driven by a cytomegalo virus promoter was constructed by subcloning an EcoRI fragment containing the p19ARF14–14 fragment from pSRaMSV hemagglutinin-ARF1–14 tk neo (kind gift of Dr. C. Sherr) into pcDNA3. The T7-Myc construct has been described before (46).

Immunostaining and Confocal Microscopy—U2OS cells were grown in 24-well plates containing coverslips. Cells were transfected either with T7-Myc (0.2 μg) alone or cotransfected with T7-Myc (0.6 μg) or with T7-Myc and p19ARF14–14 (0.6 μg) expression plasmids, using Lipofectamine 2000 reagent (Invitrogen). For the c-Myc-nucleolin co-localization experiment, cells were similarly co-transfected with plasmids expressing T7-Myc (0.2 μg) and p19ARF (0.6 μg). 24 h after transfection, the cells were fixed with methanol, blocked with 5% goat serum in phosphate-buffered saline (PBS), and probed with T7-tag monoclonal antibody (1:250 dilution) or polyclonal p19ARF Ab (1:250 dilution) or goat polyclonal C23 Ab (nucleolin; 1:100 dilution). The proteins were detected using the TRITC-conjugated anti-mouse or FITC-conjugated anti-rabbit (for p19ARF) or FITC-conjugated anti-goat (for nucleolin) secondary antibodies (all 1:200 dilution). Finally, the coverslips were washed and mounted on glass slides by using Vectashield mounting medium (Vector Laboratories). The immunofluorescence was detected, and images were taken using a CLSM 510 microscope (Zeiss) and a x63 Acrophan water immersion objective. The nucleolin antibody (C23) was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

DNA Transfections—All DNA transfections, unless and until mentioned otherwise, were used by using the calcium phosphate method as described previously (47).

CAT Assays—CAT assays were performed by the xylene extraction method (48).

Immunoprecipitation and Western Blots—The U2OS cells were harvested 48 h after transfection. The cells were washed twice with PBS and suspended in NETT250 buffer containing 200 mM Tris-HCl (pH 8.0), 0.1 mM EDTA, 250 mM NaCl, 0.5% Triton X-100 containing Complete mini, EDTA-free protease inhibitor mixture (Roche Applied Sciences) for 1 h at 4 °C. After incubation, the lysates were centrifuged at 13,000 × g for 10 min, and the supernatants were used for immunoprecipitation. p19ARF (kind gift of Dr. C. Sherr) was used for immunoprecipitation. The lysates were incubated with the antibody for 2 h at 4 °C. Protein A-Sepharose was then added, and the tubes were rocked for 1 h at 4 °C. The beads were then collected by centrifugation. Precipitates were washed three times with 400 μl of the NETT250 buffer. The bound proteins were subjected to Western blot analysis. Western blot analysis was performed by using anti-rabbit or anti-mouse Fp1 Ab (Amersham Biosciences) and total cell extracts were prepared by suspending the cells in NETT250 buffer containing Complete mini, EDTA-free protease inhibitor mixture (Roche Applied Science) for 1 h at 4 °C. After incubation, the lysates were centrifuged at 13,000 rpm for 10 min, and the supernatant was precleared by rocking with Protein G-Sepharose for 1 h at 4 °C. The precleared extracts were then subjected to immunoprecipitation with monoclonal p14ARF antibody (p1AARF/16g) or goat polyclonal C23 Ab–2 (Labvision Corp.). Cell lysates were incubated with the antibody for 2 h at 4 °C. Protein G-Sepharose beads were then added, and the tubes were rocked for 1 h at 4 °C. The beads were collected by centrifugation. Precipitates were washed three times with 400 μl of the NETT250 buffer. The bound proteins were eluted by rocking the washed beads with elution buffer (0.5 M NaCl, 0.15% SDS, and 20 mM Tris-CI, pH 8.0) for 10 min at room temperature. The eluted proteins were subjected to Western blot analysis using polyclonal c-Myc antibody (N-262; Santa Cruz Biotechnology).

Inducible Cell Line—U2OS-ARF and U2OS-ARFdi1–14 are single cell clones derived from the T-REX-U2OS cell line (Invitrogen), which were transfected with a plasmid expressing a T7 epitope-tagged p19ARF cDNA or T7-Myc cDNA for 1–14 DNA; respectively, under the control of tetracycline operator. This plasmid, T7p19ARF pcDNA4/TO, and T7p19ARFdi1–14 pcDNA4/TO were generated by cloning a PCR-amplified fragment of T7 epitope-tagged p19ARF cDNA and T7 epitope-tagged p19ARFdi1–14 cDNA into KpnI/Xhol sites of the pcDNA4/TO (Invitrogen). The respective plasmids were then transfected into T-REX-U2OS cells using LipofectAMINE 2000 (Invitrogen). 24 h after transfection, cells were split into selection medium containing 50 μg/ml zeocin (Invitrogen), and selection was continued for 2 weeks, at which time single colonies were isolated, expanded, and checked by Western blot analysis for T7p19ARF and T7p19ARFdi1–14 protein expression. A single clone that expressed the optimal level of the protein was used for the cell line. The T7p19ARF di1–14 cell line was grown in DMEM containing a 10% tetracycline system-approved fetal bovine serum (Clontech), 50 μg/ml hygromycin B (Mediatech), and 50 μg/ml zeocin (Invitrogen).

Northern Blot Analysis—U2OS-ARF and U2OS-ARFdi1–14 cells were grown in DMEM containing 50 μg/ml hygromycin and 50 μg/ml zeocin. The cells were treated with tetracycline to a final concentration of 1 μg/ml. The cells were harvested after various time periods of induction with tetracycline, and total RNA was prepared using TRIZOL (Invitrogen) as specified by the manufacturer. 20 μg of total RNA was separated on 1% agarose, 6% formaldehyde gels and transferred to Hybond-NX membranes (Amersham Biosciences) by capillary blotting. The RNA was hybridized to cDNA probes for ODC, Cul-1, and Cdk4 that had been labeled with [32P]dCTP by random priming. Northern blot analysis for ODC mRNA was done by using a 2-kb EcoRl/BamHI fragment from the pBS-ODC plasmid. For Cul-1 mRNA, Northern blot analysis was done using a...
Cul-4B (vector) (10 μg) was added to the culture medium. The incubation was continued for 6 h. After washing with cold PBS, the cells were resuspended and total cell extract was prepared as described under “Materials and Methods.” Top panel, 1.5 mg of the total cell extract was subjected to immunoprecipitation (IP) with R562 antibody (for p19ARF), and the immunoprecipitates were subjected to Western blot (WB) analysis. The blot was probed with horseradish peroxidase-linked T7 antibody (Novagen) to detect co-immunoprecipitating c-Myc. Middle and bottom panels, extracts were also tested for the expression of T7-Myc and p19ARF by probing the blot with T7 and p19ARF antibodies. B, HeLa cells were harvested, and total extracts were prepared as described under “Materials and Methods.” 2 mg of total cell extract was subjected to IP with a monoclonal antibody p14ARF/162 Ab-2 (for p14ARF), and the immunoprecipitates were subjected to Western blot analysis. The blot was probed with N-262 antibody (for c-Myc). C, p53 Mdm2− cells were harvested, and total extracts were harvested as described above. These were also tested for expression of p19ARF using HA antibody (for p19ARF), and the immunoprecipitates were subjected to Western blot analysis. The blot was probed with N-262 antibody (for c-Myc).

**RESULTS**

**ARF Associates with c-Myc**—In an attempt to investigate whether ARF targets c-Myc, we looked for a co-immunoprecipitation of c-Myc with ARF. U2OS osteosarcoma cells were transfected with a plasmid expressing T7 epitope-tagged c-Myc along with a plasmid expressing the mouse ARF protein. Also, to test the specificity of the interaction between Myc and ARF, we analyzed a mutant of ARF that lacks the N-terminal 14 residues. Mouse ARF possesses two MDM2-binding sites; one involves the N-terminal 14 residues, and the second involves the residues between 26 and 37. The N-terminal deletion mutant of ARF failed to associate with Myc. To investigate an interaction between the endogenous c-Myc and ARF, we immunoprecipitated HeLa whole-cell extracts (2 mg) with a monoclonal anti-
body against human ARF or the hemagglutinin epitope. The immunoprecipitates obtained with the human ARF antibody, but not the hemagglutinin antibody, contained c-Myc (Fig. 1B).

To investigate whether the ARF/Myc interaction requires MDM2, we performed a co-immunoprecipitation experiment using extracts from p53\(^{−/−}\) Mdm2\(^{−/−}\) mouse embryonic fibroblasts (16, 50). The co-immunoprecipitation experiment provided evidence for an interaction between endogenous ARF and Myc in p53\(^{−/−}\) Mdm2\(^{−/−}\) cells (Fig. 1C), suggesting that ARF can associate with c-Myc independently of MDM2 and p53. The c-Myc protein from p53\(^{−/−}\) Mdm2\(^{−/−}\) cells migrated as a doublet, and the upper band was specifically detected in the immunoprecipitate with ARF antibody. It is therefore possible that ARF binds to a modified form of c-Myc in those cells.

**ARF Relocalizes c-Myc**—We then sought to determine whether ARF has any effect on the localization of the Myc protein, because ARF has been shown to relocalize MDM2 to the nucleolus. To investigate subcellular localization of c-Myc and ARF by immunofluorescence, U2OS cells grown on coverslips were transfected with the plasmid expressing T7 epitope-tagged c-Myc alone or along with the ARF expression plasmid. Following transfection, the coverslips were subjected to immunostaining using T7 antibody for Myc and ARF antibody to detect the localization of ARF. Fluorescent conjugated secondary antibodies (TRITC for c-Myc and FITC for ARF) were used for visualization. The subcellular localization of Myc and ARF were visualized using a confocal microscope. As expected, Myc was found mainly in the nucleoplasm when expressed alone. However, coexpression of ARF resulted in a relocalization of Myc from the nucleoplasm to the nucleolus (Fig. 2). The relocalization was seen in all cells that were expressing ARF and c-Myc. To confirm that ARF relocalizes Myc to the nucleolus, the cells transfected with ARF and Myc expression plasmids were also probed with antibody against nucleolin, a specific marker for the nucleolus. Clearly, in the presence of ARF, the Myc protein co-localized with nucleolin (Fig. 2). Because the mutant harboring deletion of the N-terminal 14 residues failed to associate with Myc, we predicted that this mutant would be impaired in its ability to relocalize Myc to the nucleolus. When the mutant ARF was coexpressed with Myc, there was no colocalization. The mutant ARF localized in the nucleolus, and the Myc protein was detected in the nucleoplasm with very little or no overlap (Fig. 2). The results are consistent with the notion that ARF binds to Myc and relocalizes Myc to the nucleolus.

**ARF Inhibits c-Myc-activated Transcription**—Myc is a nuclear protein. Association of Myc with ARF and the ARF-mediated relocalization are expected to result in an inhibition of the function of Myc. Therefore, we sought to determine whether the transcriptional activity of Myc is inhibited by the expression of ARF. We employed the Gal-Myc fusion protein (51), which allows tethering of the c-Myc protein to a promoter containing the Gal4-binding sites. For a control, a Gal-HNF3 fusion protein was used to stimulate transcription from a promoter containing the Gal4-binding sites. HNF3 is a tissue-specific transcription factor that does not associate with ARF (not shown). Plasmids expressing Gal-Myc or Gal-HNF3 were transfected into U2OS cells along with a reporter CAT gene containing five Gal4-binding sites (G5bCAT) in the presence or absence of the ARF expression plasmid. A plasmid expressing β-galactosidase was included in all transfection mixtures to normalize for the transfection efficiencies. Both Gal-Myc and Gal-HNF3 stimulated transcription from the reporter gene. Expression of ARF caused a significant inhibition of the Gal-Myc-activated transcription, whereas the transcription activated by Gal-HNF3 was only marginally affected by the expression of ARF (Fig. 3). The results are consistent with an ARF/Myc interaction and relocalization of c-Myc to the nucleolus. Also, we compared the wild-type ARF and the N-terminal deletion mutant of ARF (ARF\(^{d1−14}\)) for their ability to inhibit transcription activated by Gal-Myc. Clearly, the mutant ARF, which fails to associate with c-Myc and relocalize c-Myc, was deficient in inhibiting Gal-Myc-activated transcription (Fig. 3A). Moreover, we observed that the ARF inhibition of the c-Myc-activated transcription is independent of p53, since ARF could efficiently inhibit transcriptional activity of Gal-Myc in the SAOS2 (p53-negative) cells (Fig. 3B).

**ARF Inhibits c-Myc-induced Genes**—We then sought to investigate whether the endogenous c-Myc-induced genes are inhibited by ARF. Also, if regulation of the Myc-induced genes is important for the G\(_1\) arrest function of ARF, we expect to see inhibition of the Myc-induced genes before inhibition of S phase progression. To investigate these possibilities, we constructed a cell line (U2OS-ARF) in which ARF could be expressed inducibly by the addition of tetracycline in the culture medium. U2OS-derived cell line (Invitrogen) was used for this purpose. The addition of 1 μg/mL tetracycline induced expression of ARF that caused G\(_1\) and G\(_2\) arrest (not shown). To analyze the effect of ARF on the Myc-induced genes, the U2OS-ARF cells were induced with tetracycline for different time periods (12, 18, 24, and 36 h). Cells were harvested, and total RNA was prepared. The RNAs were subjected to Northern blot analysis. The blots were probed for the levels of Myc-induced genes: ODC, Cul-1, and Cdk4. Expression of ARF in the U2OS-ARF cells resulted in a dramatic reduction of the levels of both ODC and Cul-1 mRNAs (Fig. 4, left). The inhibition of the ODC and Cul-1 mRNAs was detected before a complete arrest of the cell cycle (Fig. 4, right), suggesting that ARF inhibits expression of the Myc-induced gene before inhibiting progression to S phase. We did not observe inhibition of Cdk4 mRNA. It is likely that the level of Cdk4 mRNA can be maintained in a Myc-independent pathway. To investigate a link between the Myc/ARF interaction and the inhibition of ODC mRNA level, we constructed a cell line that expresses the mutant ARF (ARF\(^{d1−14}\)) in an inducible manner. The two cell lines expressing the wild-type ARF and mutant ARF were compared side-by-side for binding to Myc and an inhibition of ODC mRNA (Fig. 5). As expected, the endogenous c-Myc in U2OS cells co-immunoprecipitated with ARF when ARF was expressed, but no such interaction was observed with the mutant ARF (ARF\(^{d1−14}\)). This suggests that the ARF/Myc interaction affects the level of the Myc mRNA.
with the wild-type ARF but not with the mutant ARF (Fig. 5A). Consistent with that, the level of ODC mRNA was not significantly inhibited in the mutant ARF-expressing cells after the addition of tetracycline (Fig. 5B).

Control of c-Myc Activity by Endogenous ARF—We have demonstrated that ARF could relocalize c-Myc to the nucleolus. Since these studies were carried out under conditions of overexpression, we were interested in investigating whether the endogenous ARF protein would also relocalize Myc to the nucleolus. Since the p53/H11002 Mdm2/H11002 MEFs are known to express a higher level of endogenous ARF, we decided to look at the subcellular localization of Myc in these cells. As a comparison, we also looked at the subcellular localization of Myc in p53/ Mdm2/ ARF− MEFs. p53/ Mdm2/ and p53/ Mdm2/ ARF− MEFs, grown on coverslips, were infected with recombinant adenovirus expressing c-Myc (Ad-Myc). Following infection, the coverslips were subjected to immunostaining using a monoclonal antibody against Myc and a polyclonal ARF antibody to detect localization of ARF. Fluorescent conjugated secondary antibodies (FITC for c-Myc and TRITC for ARF) were used for visualization. In the p53/ Mdm2/ ARF− MEFs, Myc was mainly nuclear (Fig. 6A, bottom panel), whereas in the p53− Mdm2/ MEFs, we found that Myc indeed colocalized with the endogenous ARF protein in the nucleolus (Fig. 6A, top panel), suggesting that endogenous ARF is capable of relocalizing Myc to the nucleolus.

We further looked to see whether the c-Myc-activated genes were inhibited by endogenous ARF. To look for an effect of the endogenous ARF on the Myc-activated gene ODC, we compared p53/ Mdm2/ and p53− Mdm2/ ARF− MEFs. The MEFs were infected either with a control adenovirus (Ad-TA) or an adenovirus expressing c-Myc (Ad-Myc). Total RNA was prepared from the infected cells and subjected to Northern blot analysis to check for the levels of ODC mRNA. Fig. 6B shows that expression of Myc in the p53/ Mdm2/ MEFs, which are known to express high levels of endogenous ARF, resulted in only a negligible increase in the levels of ODC mRNA (the lane corresponding to Myc-infected p53/ Mdm2/ MEFs contained more RNA as judged by the rRNA loading control). However, in the p53− Mdm2/ ARF− MEFs, which lack functional ARF, expression of Myc resulted in a significant increase in the levels of ODC mRNA (Fig. 6B). Since expression of Myc resulted in a significant increase in levels of ODC in cells lacking...
ARF compared with cells having endogenous ARF, the results are consistent with the notion that endogenous ARF inhibits c-Myc function.

siRNA-mediated Knockdown of ARF Increases Expression of the Myc-induced Gene ODC—To investigate whether the endogenous ARF inhibits function of endogenous c-Myc, we used siRNA to knock down ARF. We used MEFs from p53<sup><sup>-/-</sup></sup> Mdm2<sup><sup>-/-</sup></sup> mouse embryos. The hypothesis was that the endogenous ARF would normally repress c-Myc activity independently of p53 and MDM2. siRNA-mediated reduction in the levels of ARF in

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**Fig. 4. ARF expression inhibits c-Myc-activated genes.** U2OS-ARF cells were induced with tetracycline (1 mM). Cells were harvested at the indicated time points, and total cellular RNA (20 μg) was subjected to Northern blot analysis (left). The blots were probed with <sup>32</sup>P-labeled DNA probe derived from Cul1, ODC, and Cdk4 cDNA as described under “Materials and Methods.” 100 μg of total cell extract prepared from cells harvested at the indicated time points was subjected to Western blot analysis by probing the membrane with the R562 antibody (for p19<sup>ARF</sup>). Right, plots showing the percentages of inhibition of S phase, expression of Cul1 mRNA, ODC mRNA, and Cdk4 mRNA after induction of U2OS-ARF cells with tetracycline. The percentage of cells in S phase was determined by fixing and staining cells collected at the indicated time points with propidium iodide (25 μg/ml) and determining the DNA content with FACSCalibur.

**Fig. 5.** Mutant ARF (d1–14) fails to bind the endogenous c-Myc in U2OS cells and to inhibit ODC mRNA. A, U2OS-ARF and U2OS-ARFd1–14 cells were induced with tetracycline (1 mM). 20 h after induction, cells were harvested, and total cell extract was prepared as described under “Materials and Methods.” 2 μg of the total cell extract was subjected to immunoprecipitation with R562 antibody (for p19<sup>ARF</sup> or p19<sup>ARFd1–14</sup>). The immunoprecipitates were subjected to Western blot analysis by probing the membrane with C33 antibody (for c-Myc). B, U2OS-ARF and U2OS-ARFd1–14 cells were induced with tetracycline (1 mM). Cells were harvested at the indicated time points, and total cellular RNA (20 μg) was subjected to Northern blot analysis. The blots were probed with <sup>32</sup>P-labeled DNA probe derived from ODC and Cdk4 cDNA.
those cells would result in a derepression of c-Myc activity. In order to test this model, p53/Mdm2 and p53/Mdm2/ARF MEFs were infected with either a control or c-Myc-expressing recombinant adenoviruses. 18 h after infection, cells were processed for immunostaining and confocal microscopy as described under “Materials and Methods.” A monoclonal antibody against c-Myc (C-33 Ab, 1:50 dilution; Santa Cruz Biotechnology) and a polyclonal antibody against p19ARF (R562; 1:200 dilution; GeneTex) were used to detect the Myc and ARF proteins, respectively. B, p53/Mdm2− and p53/Mdm2/ARF MEFs were infected with control (Ad-TA) or c-Myc (Ad-Myc)-expressing adenoviruses. 18 h after infection, cells were harvested, and total RNA was extracted using TRIZOL. 20 μg of total RNA was subjected to Northern blot analysis to detect the levels of ODC mRNA. Levels of 28 and 18 S rRNA are shown as loading controls.

Myc-ARF Interaction Inhibits the Functions of Myc

36704

Fig. 6. Regulation of Myc function by endogenous p19ARF. A, p53/Mdm2− and p53/Mdm2/ARF MEFs were grown on coverslips and infected with either a control or c-Myc-expressing recombinant adenoviruses. 18 h after infection, cells were processed for immunostaining and confocal microscopy as described under “Materials and Methods.” A monoclonal antibody against c-Myc (C-33 Ab, 1:50 dilution; Santa Cruz Biotechnology) and a polyclonal antibody against p19ARF (R562; 1:200 dilution; GeneTex) were used to detect the Myc and ARF proteins, respectively. B, p53/Mdm2− and p53/Mdm2/ARF MEFs were infected with control (Ad-TA) or c-Myc (Ad-Myc)-expressing adenoviruses. 18 h after infection, cells were harvested, and total RNA was extracted using TRIZOL. 20 μg of total RNA was subjected to Northern blot analysis to detect the levels of ODC mRNA. Levels of 28 and 18 S rRNA are shown as loading controls.
DISCUSSION

Expression of the ARF gene is induced by a variety of oncogenic stresses. Included are adenovirus E1A, Ras, c-Myc, E2F1, and deregulated β-catenin (45, 55–62). Following activation by the oncopgenes, ARF stabilizes p53 and activates the tumor suppression pathways of p53, which leads to apoptosis or a senescence-like phenotype in cells undergoing oncogenic stress. For example, E1A and c-Myc were shown to induce apoptosis, whereas Ras and β-catenin were shown to induce growth arrest and a senescence-like phenotype. Apoptosis and senescence preclude oncogenic transformation. Thus, ARF elicits safeguard pathways that protect cells from oncogenic activation. Although ARF can exert its effect via stabilization of p53, recent studies have indicated that the activation and stabilization of p53 might not be the only mechanism by which ARF functions as a tumor suppressor (23–25). Results presented in this study suggest that the c-Myc-activated pathway of cell proliferation is directly targeted and counteracted by ARF, independently of p53 status. In many cancers, both the p53-dependent and the p53-independent safeguard mechanisms are evaded, since the expression of the ARF gene is found to be extinguished by hypermethylation (8).

We observed that expression of ARF resulted in an inhibition of the c-Myc-activated genes ODC and Cul-1, which have been implicated in the pathways by which c-Myc stimulates cell proliferation. We failed to detect an inhibition of the Cdk4 mRNA levels. It is possible that Cdk4 expression can be maintained by a Myc-independent pathway. In this regard, it is noteworthy that the Myc induction of the telomerase gene was shown to be deregulated in many immortalized cells (63). We confirmed that the effect of ARF on the c-Myc-activated genes ODC and Cul-1 was not indirectly caused by a cell cycle inhibition, since we show that these events occur prior to the inhibition of S phase progression. We investigated the effect of

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**FIG. 7.** Endogenous p19ARF inhibits c-Myc activity. p53−/−Mdm2−/−MEFs were infected with either a control retrovirus or a retrovirus-expressing siRNA against p19ARF. Infection was done as described under “Materials and Methods,” and the infected cells were either processed for preparation of total cell extract or total cellular RNA, as described under “Materials and Methods.” A, 150 μg of total cell extracts was assayed for expression of p19ARF by Western blot analysis by probing the blot with R562 antibody (for p19ARF; GeneTex). The same blot was also probed with a M2 antibody (for Cdk2; Santa Cruz Biotechnology), as a loading control. B, 20 μg of total cellular RNA was analyzed by Northern blot analysis for levels of ODC by probing the membrane with a 32P-labeled probe against ODC. The levels of 28S and 18S rRNA are shown as loading controls.

**FIG. 8.** ARF expression blocks c-Myc-induced S phase progression. A, HaCat cells were grown on coverslips and starved for 72 h by growing in medium without serum. Cells were then infected with the indicated combination of recombinant adenoviruses. 12 h after infection, BrdUrd (10 μM) was added to the medium, and the cells were grown for an additional 6 h. Cells were then fixed and immunostained using a monoclonal antibody against BrdUrd and an FITC-labeled secondary antibody. 100 cells were counted on three separate coverslips and scored for incorporation of BrdUrd, and the average percentage of BrdUrd-positive cells is represented in the plot. B, 10.1 Myc-ER cells were grown on coverslips and starved for 70 h by growing in medium containing no serum. Cells were then infected with the indicated combinations of recombinant adenoviruses. Immediately after infection, 4-HT (1 μM) was added to the medium. BrdUrd incorporation and immunostaining was done as above. The plot represents an average percentage of BrdUrd-positive cells scored in 100 cells each from three independent coverslips.

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**DISCUSSION**

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endogenous ARF on Myc by analyzing the subcellular localization of Myc and the levels of ODC mRNA in ARF+/+ and ARF−/− background and found that the endogenous ARF protein could regulate Myc function. More significantly, knocking down the levels of endogenous ARF in p53−/− Mdm2−/− MEFs using siRNA technology led to an increase in the levels of ODC mRNA, highlighting the physiological relevance of the ARF/Myc interaction. The c-Myc protein is a potent activator of S phase, and it can stimulate entry into S phase in the absence of growth factors (54). We observed that expression of ARF inhibited c-Myc-induced S phase in cells lacking p53 or expressing a mutant p53. These observations are consistent with the notion that c-Myc is a bona fide target of ARF. We propose that ARF forms a physical complex and sequesters c-Myc in the nucleolus to impede its function.

Overexpression of c-Myc has been shown to induce apoptosis in the absence of an adequate level of survival factors (30). It was shown that c-Myc induces apoptosis by increasing the expression of ARF (45), which increases the level and activity of p53. Thus ARF acts in a feedback loop to eliminate cells expressing high levels of Myc through a p53-dependent pathway (Fig. 9). In the presence of survival factors, c-Myc does not induce apoptosis; it increases cell proliferation. Therefore, c-Myc is oncogenic in the presence of an adequate level of the survival factors (30). Our observations imply that ARF can also directly attenuate the oncogenic activity of c-Myc in a p53-independent manner. It is noteworthy that ARF can target the E2F family of transcription factors (25–27). Therefore, a similar model can be envisioned for the E2F family factors as well.

In this scenario, in the absence of survival factors, ARF enhances function of p53 to induce apoptosis. As shown above, ARF can also attenuate the proliferation signals by sequestering the key mediators such as c-Myc and E2F1, and this attenuation would be particularly important in tumor suppression when there is an adequate supply of the survival factors. A dual function of ARF in both directions (curbing mitogenic signals and activating apoptotic pathways) would offer a more effective mechanism of tumor suppression. We suggest that the interplay between ARF and the proliferation factors is one way to maintain a delicate control of cellular proliferation. If the level of c-Myc exceeds the level required for normal cellular proliferation, a safeguard mechanism is activated whereby Myc increases ARF level, which in turn down-regulates Myc. We predict that c-Myc must overcome ARF inhibition (probably by titrating it out) in order to bring about oncogenesis.

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