A tumor suppressor gene product, ARF, sensitizes cells to apoptosis in the presence of appropriate collateral signals. In this study, we analyzed the mechanism of ARF-dependent apoptosis and demonstrated that ARF induces mitochondria-dependent apoptosis in p53 wild-type, ARF/p16-null cells. We also found that ARF evokes cytochrome c release from mitochondria, decreases mitochondrial membrane potential, and activates procaspase-9 to induce apoptosis. Our findings suggest that this apoptotic cellular modulation is brought about by up-regulation of the proapoptotic Bcl-2 family proteins Bax and Bim and down-regulation of antiapoptotic Bcl-2 in mitochondrial fractions. Additionally, ARF seems to down-regulate Bcl-2 in a p53-dependent manner while up-regulating Bax/Bim via a p53-independent pathway.

Apoptosis is an essential physiological mechanism for the selective elimination of cells (1). It plays an integral role in a variety of biological events including morphogenesis, cell turnover, and removal of harmful (especially malignant-transformed) cells. Apoptotic machinery can also be activated by a variety of stimuli, such as pathological reagents, that cause various kinds of stress.

The p53 tumor suppressor gene is the central integrator of the cellular response to stress, e.g. DNA damage, oncogenic transformation, and growth factor withdrawal (2, 3). The cell cycle regulation and the DNA repair functions of p53 are mainly executed by transactivation of p53 response genes such as p21

\(^{\text{Cip1/Waf1}}\) (4) and GADD45 (5). Similarly, for induction of apoptosis, the importance of regulation by p53 has been demonstrated in many studies (2). However, the pathways whereby p53 leads to execution of the apoptosis pathway need to be better characterized because even dependence of p53-induced apoptosis on cytochrome c release from mitochondria has been controversial (6, 7).

The \(\text{p53}^{\text{null}}\)/ARF locus is second only to \(\text{p53}^{\text{null}}\) in the frequency of its disruption in human cancer (8). The \(\text{INK4a/ARF}^{\text{null}}\) locus encodes p16\(^{\text{NTrc}}\), an important cyclin-dependent kinase inhibitor, and a second protein translated in an alternative reading frame designated as ARF (9). Mice lacking ARF alone are highly cancer-prone, and ARF-null mouse embryo fibroblasts are transformed by oncogenic Ras alone (10), indicating that ARF is a bona fide tumor suppressor. ARF binds directly to MDM2, sequestering it in the nucleus and enabling transcriptionally active p53 to accumulate in the nucleoplasm (11–14), as well as triggering p53-dependent growth arrest in cell cycle G\(_1\) and G\(_2\) phases. In the presence of appropriate collateral signals, ARF sensitizes cells to apoptosis (15, 16). Of particular interest has been the discovery that several oncogenes can induce stabilization of p53 by up-regulating the activity of ARF (15–18). Although ARF activation and p53 stabilization alone are not sufficient to induce apoptosis in all cell types, up-regulation of p53 in this way strongly sensitizes cells to die in response to genotoxic stresses or additional apoptotic signals induced by oncogenes themselves (15, 16). However, the exact mechanism by which ARF induces apoptotic cell death has not been clearly demonstrated. We therefore studied the mechanism by which the ARF tumor suppressor induces cell death to bring new insight into the cellular self-defense machinery against insult.

Here, we demonstrated that ARF induces mitochondria-dependent apoptosis in p53 wild-type, ARF/p16-null cells in the absence of growth factors. This apoptotic cellular modulation appears to be brought about by an increase of two proapoptotic Bcl-2 family proteins, Bax and Bim, in mitochondrial fractions via a p53-independent pathway. Alternatively, antiapoptotic Bcl-2 is down-regulated in mitochondrial fractions in a p53-dependent manner.

MATERIALS AND METHODS

Reagents and Antibodies—Anti-p19ARF rabbit polyclonal antibody (R562) was purchased from Abcam (Cambridge, United Kingdom). Anti-Bax rabbit polyclonal antibody was from Upstate Biotechnology (Lake Placid, NY). Anti-p53 mouse monoclonal antibody (clone pAb421) was from Oncogene Research Products (Cambridge, MA). Anti-Bim rabbit polyclonal antibody was from Millennium Biotechnology (Ramona, CA). Anti-Bcl-2 mouse monoclonal antibody (clone C-2), anti-p21\(^{\text{Cip1/Waf1}}\) mouse monoclonal antibody (clone F-5), and anti-Bad mouse monoclonal antibody (clone C-7) were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-cytochrome c mouse monoclonal antibody (clone 7H8.2C12), anti-Bcl-X\(_M\) mouse monoclonal antibody (clone 2H12), anti-caspase-3 mouse monoclonal antibody (clone 46), and anti-Bid rabbit polyclonal antibody were from BD PharMingen. Anti-caspase-8 mouse monoclonal antibody (clone 12F5) was from ALEXIS Biochemicals (San Diego, CA). Anti-caspase-9 mouse monoclonal antibody (clone 5B4) was from MBL (Nagoya, Japan). Anti-trifunctional protein (19) serum was prepared by rabbit immunization and affinity-selection with purified trifunctional protein. The broad-spectrum caspase inhibitor benzoxycarbonyl-Val-Ala-Asp-fluoromethyl ketone (zVAD-fmk)\(^{1}\) was purchased from Enzyme Systems Products (Livermore, CA). Other

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\(^{1}\) The abbreviations used are: zVAD-fmk, benzoxycarbonyl-Val-Ala-Asp-fluoromethyl ketone; FBS, fetal bovine serum; FACS, fluorescence-activated cell-sorting.
Fig. 1. ARF induces apoptosis in cells cultured in low-concentration serum media. Cells (3 × 10^6) were seeded onto a coverslip and infected with retroviruses produced by pRS-MSV-tk-CD8 containing hemagglutinin-tagged p19ARF cDNA. Cells were cultured with 10% (v/v) FBS for 24 h, followed by apoptosis induced by serum starvation as described under “Cells and Cell Culture.” A, phase-contrast microscopic analysis of control virus-infected cells (mock) and ARF virus-infected cells (ARF). B, laser confocal microscopic analysis of ARF virus-infected cells. Fixation, permeabilization, and staining were performed as described under “Materials and Methods.” Top panels, cells starved for 24 h with 0.1% (v/v) FBS-containing media 24 h after infection; bottom panels, cells fed with 10% (v/v) FBS-containing media 48 h after infection. The panels show nuclear DNA staining by propidium iodide (PI, first column, red), ARF fluorescence (second column, green), differential interference contrast (DIC) image (third column, gray scale), and propidium iodide-ARF-differential interference contrast merge (fourth column).

biochemical reagents were purchased from Sigma-Aldrich or Wako (Osaka, Japan).

Cells and Cell Culture—A p53 wild-type, ARF/p16-null fibroblast cell line, NIH3T3, was used in experiments to assess the induction of apoptosis by ARF expression using a retrovirus because p53 is considered to be an important downstream effector of tumor suppression mediated by ARF (9, 10). The cells were routinely maintained with Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (FBS). To induce apoptosis, cells were washed twice with phosphate-buffered saline 24 h after virus infection and then cultured with 0.1% (v/v) FBS-containing media.

Retrovirus Production and Infection—A retrovirus vector for virus production, pMSCV-IRE-\text{GFP}, was a generous gift from Dr. Robert G. Hawley (University of Toronto). Virus production and cell infection were performed using retroviral helper and vector plasmids (20) provided by Charles Sawyer (University of California Los Angeles). Cells from the human kidney 293T cell line (21) were from David Baltimore (California Institute of Technology, Pasadena, CA). The following cDNAs were cloned into pMSCV-IRE-\text{GFP} or pRS-MSV-tk-CD8 plasmids for production of recombinant retroviruses: mouse ARF (9), mouse p19ARF (22), human Bcl-2 (23), and human Bcl-xL (24).

Morphological Analysis of Apoptosis—Cells were observed using a phase-contrast microscope to assess apoptotic morphological changes and treated with Hoechst 33258, a DNA-staining dye, to detect the morphological characteristics of apoptotic nuclei, namely, condensation (early stage of nuclear apoptosis) and fragmentation (late stage of nuclear apoptosis) (25), after fixation with 2% paraformaldehyde.

Immunofluorescence—Fixation was performed with 3.7% (v/v) formaldehyde/1% phosphate-buffered saline for 30 min, and cell permeabilization was done with 0.1% (v/v) Triton X-100/1% phosphate-buffered saline for 5 min at room temperature. Cells were then stained for 1 h with anti-p19ARF rabbit polyclonal antibody (R562), followed by a 30-min exposure to anti-rabbit Ig antibody conjugated with fluorescein isothiocyanate (Sigma). DNA was visualized with propidium iodide. Analysis by confocal laser microscopy was performed with an LSM410 system (Carl Zeiss).

Intersensosomal DNA Fragmentation Assay—Retrovirus-infected cells (2 × 10^6) were lysed for 10 min on ice in 100 μl of hypotonic lysis buffer (10 mM Tris, pH 7.5, 10 mM EDTA, pH 8.0, and 0.5% Triton X-100). After centrifugation for 10 min at 14,000 × g, the supernatant was transferred to a new tube and incubated for 30 min at 50 °C in the presence of 0.2 mg/ml RNase A and 0.2 mg/ml proteinase K. DNA was precipitated with 1.2 volumes of isopropanol and 2 volume of 3 M NaCl overnight at −20 °C. After centrifugation at 14,000 × g, the pellets were dried, dissolved in 20 μl of Tris-EDTA, and then analyzed by gel electrophoresis in 2% agarose and ethidium bromide staining.

Analysis of Sub-G_{1}, G, Fraction—After retrovirus infection and serum starvation, floating cells were collected by centrifugation, and attached cells were collected from plates using phosphate-buffered saline containing 5 mM EDTA. The collected cells were suspended in 500 μl of DNA staining buffer (0.1% (w/v) sodium citrate, 50 μg/ml propidium iodide, 1 μg/ml RNase A, and 0.1% (v/v) Triton X-100) and incubated at room temperature for 30 min. Analysis of the sub-G_{1}, G, fraction was performed using a FACScan flow cytometer (Becton Dickenson). 15,000 cells were analyzed for each sample, and quantitation of cell cycle distribution was performed using Cell Quest software (Becton Dickenson).

Cell Fractionation—2 × 10^6 cell aliquots were suspended in 0.5 ml of buffer (20 mM HEPES, pH 7.5, 10 mM KCl, 1.5 mM MgCl_2, 1 mM EDTA, 1 mM EGTA, 1 mM diethytoethyl, 0.1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml each of leupeptin and aprotonin) containing 250 mM sucrose. The cells were homogenized by 10 strokes in a Dounce homogenizer, and homogenates were centrifuged twice at 750 × g for 5 min at 4 °C to collect nuclei and debris. The supernatant was centrifuged at 10,000 × g for 15 min at 4 °C to collect the heavy membrane fraction. The resulting supernatant was finally centrifuged at 100,000 × g for 1 h at 4 °C. The final supernatants are referred to as cytosol fractions, and the pellets were collected as light membrane fractions.

Western Blotting—Frozen cell pellets were lysed in ice-cold TWEEN 20 lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM EDTA, 2.5 mM ethylene-glycolterhaetic acid, 0.1% Tween 20, 1 mM phenylmethylsulfonyl fluoride, 0.4 unit/ml aprotonin, and 10 μg/ml leupeptin and sonicated. Protein concentration was determined by BCA Protein Assay kit® (Pierce). Samples were subjected to electrophoresis on denaturing polyacrylamide gels containing SDS and transferred to Immobilon-P® polyvinylidene difluoride membranes (Millipore, Bedford, MA). Filters were then blocked with 5% bovine serum albumin in Tris-buffered saline for 1 h at room temperature and subsequently probed for 1 h at room temperature with 0.5 μg/ml or 1,2,000 dilution of primary antibodies. After five washes in Tris-buffered saline containing 0.2% (v/v) Tween 20, filters were incubated with peroxidase-conjugated secondary antibodies at a final dilution of 1:2,000. After five washes as described above, protein detection was visualized by ECL (Amersham Biosciences) according to the manufacturer’s protocol.

RESULTS

ARF Induces Apoptosis in p53 Wild-type Cells—In previous experiments, we were not able to detect apoptotic changes in ARF-expressing cells by terminal deoxynucleotidyl transferase-mediated nick end labeling assay in the presence of 10% serum (9). However, several earlier reports indicated that oncogenic hyperproliferative signals induced apoptosis via an ARF-p53 pathway, especially in serum-free culture conditions (26), which prompted us to examine whether retrovirus-mediated ARF expression in a serum-free culture condition induced apoptosis. Cells were observed with a phase-contrast microscope 24 h after serum deprivation. Fig. 1A shows that the control retrovirus-infected cells displayed a spindle cell shape.
Fig. 2. ARF modulates expression of pro- and antiapoptotic Bcl-2 family proteins. The cells were infected with retroviruses produced by a vector alone (mock) or a vector encoding p19ARF (ARF). Serum starvation was performed as described under “Materials and Methods,” and cells were collected at the indicated time points after serum starvation. A, 30 μg of protein were subjected to SDS-PAGE analysis, and signals for specific proteins were detected by direct immunoblotting using antibodies to Bax, Bim, Bcl-2, p53, p21Cip1/Waf1, ARF, and β-tubulin as indicated to the left. Results were representative of three independent experiments. Bim EL (extra long), Bim L (long), and Bim S (short) are alternative splicing products coded by the Bim gene (43). B, 8 h after starvation, collected cells were fractioned as described under “Materials and Methods.” 30-μg fractions (Total, total cell lysate; Pellet, debris/nuclei; LM, light membrane fraction; HM, heavy membrane fraction; Cytosol, cytosol fraction) were analyzed by Western blot with anti-Bim antibody, anti-Bax antibody, anti-Bcl-2 antibody, anti-p53 antibody, anti-β-tubulin antibody, anti-mitochondrial trifunctional protein complex serum, and Anti-p19ARF antibody. triE L, a large subunit of mitochondrial trifunctional protein (19).
virus reports demonstrate the redistribution of Bax from the cytosol to mitochondria after induction of apoptosis (28). In this experiment, ARF also increased the amount of Bax in mitochondrial fractions before and after serum starvation, but a decrease of Bax in the cytosol was not noticeably observed after serum starvation in ARF-expressing cells. Taken together, these results suggest that Bax accumulation in mitochondrial fractions is caused by Bax up-regulation by ARF and is not the outcome of redistribution from the cytosol. Protein loading was confirmed by β-tubulin and the large subunit of the mitochondrial fatty acid β-oxidation multienzyme complex, which was purified from the mitochondrial heavy membrane fraction (19). ARF was detected only in the Total and Pellet fractions, consistent with previous reports (9, 13), indicating that modulation of Bcl-2 family proteins seems not to be a result of direct protein interaction.

Antiapoptotic Bcl-2 Family Proteins Abrogate ARF Cytotoxicity—Next, we confirmed the modulating role of proapoptotic and antiapoptotic Bcl-2 family proteins in mitochondrial fractions of ARF-expressing cells. Co-infection experiments established that antiapoptotic Bcl-2 and Bcl-xL could block apoptosis induced by ARF in ARF/p16-null cells (Fig. 3). The loss of DNA content, a well-known marker of apoptosis, was measured by flow cytometry using propidium iodide staining. Fig. 3 shows the original fluorescence results obtained from representative experiments. Co-infection with Bcl-2 or Bcl-xL viruses effectively diminished the apoptotic fraction in ARF-expressing cells (Fig. 3A).

Inhibition of ARF-induced apoptosis by antiapoptotic Bcl-2 family molecules was further analyzed in Fig. 3B. The percentage of cells displaying apoptotic nuclei was significantly decreased by Bcl-2 or Bcl-xL virus infection in ARF virus-infected cells (ARF, 27 ± 3%; ARF/Bcl-2, 7 ± 3%; ARF/Bcl-xL, 7 ± 1%).

**ARF Overexpression Induces Mitochondrial Dysfunction—** The fact that ARF expression modulated Bcl-2 family protein amounts in mitochondrial fractions and induced apoptosis prompted us to investigate cytochrome c release from mitochondria and mitochondrial membrane potential. In order to detect whether cytochrome c release is involved in ARF-induced apoptosis, we examined its distribution in ARF-expressing cells 8 h after serum starvation by cell fractionation experiments. Cellular fractions, including cytosol fraction, light membrane fraction, and total cell lysate proteins, were prepared, and cytochrome c protein was detected by immunoblotting (Fig. 4A). Cytochrome c was not released from mitochondria in noninfected and mock-infected cells after serum starvation. However, a significant amount of cytochrome c was detected in the cytosol fraction of ARF-infected cells (Fig. 4A, lane 9). Although cytochrome c was slightly induced by ARF in these cells (Fig. 4A, lane 15), this release was not nonspecific because TriE L and ARF were undetectable in the cytosol (Fig. 4A, lane 9).

We next investigated the role of mitochondrial membrane potential in ARF-induced apoptosis. Mitochondrial membrane potential was assessed 12 h after serum starvation by staining with the mitochondrion-selective dye, MitoTracker®. ARF-expressing cells exhibited substantial mitochondrial depolarization, as evidenced by loss of MitoTracker® staining (Fig. 4B). Co-expression of antiapoptotic Bcl-2 family proteins, Bcl-2 and Bcl-xL, effectively restored the depolarization of the mitochondria induced by ARF overexpression (Fig. 4B). These results suggest that mitochondrial dysfunction (the release of cytochrome c into the cytosol and down-modulation of mitochondrial membrane potential) plays a pivotal role in ARF-induced apoptosis and is caused by modulation of Bcl-2 family proteins in mitochondria.

**Activation of Caspases Is Required for ARF-induced Apoptosis—** The central component of apoptosis is a proteolytic system involving a family of proteases called caspases (29). A large body of genetic and biochemical evidence supports a cascade model for effector caspase activation: a proapoptotic signal culminates in an activation of initiator caspases, which, in turn, activate effector caspases.

To analyze whether caspase cascades are required for ARF-induced apoptosis, we treated retrovirus-infected cells with a general caspase inhibitor (zVAD-fmk). As shown in Fig. 5A, the addition of up to 100 μM zVAD-fmk almost completely prevented an apoptotic morphological change of nuclei in ARF-expressing cells. Different initiator caspases mediate distinct sets of signals, e.g. caspase-8 is associated with apoptosis involving death receptors, and caspase-9 is involved in death induced by cytotoxic agents (29). Moreover, activation of caspase-9 requires cytochrome c, which is released from mitochondria, as well as deoxyadenosine triphosphate, indicating that caspase-9 activation requires multiple cofactors. Results indicating mitochondrial dysfunction induced by ARF prompted us to analyze the activation of caspases in ARF-expressing cells that induce apoptosis after serum starvation. Pro-caspase-9 cleavage was observed in ARF-expressing cells as early as 8 h after starvation but was not observed in control

**Flow Cytometry Using Propidium Iodide Staining—** Fig. 3 shows content, a well-known marker of apoptosis, was measured by independent experiments.
ARF induces apoptosis in the absence of growth factors

Fig. 4. ARF-induced apoptosis is dependent on a mitochondrial pathway that can be abrogated by Bcl-2 and Bcl-xL. A, Western blot analysis of cytochrome c. Total, total cell lysates; Pellet, debris/nuclei; LM, light membrane fraction; HM, heavy membrane fraction; Cytosol, cytosol fraction. Lanes 1, 4, 7, 10, and 13, noninfected cells; lanes 2, 5, 8, 11, and 14, mock-infected cells; lanes 3, 6, 9, 12, and 15, ARF-infected cells. Cells were collected 8 h after serum starvation. 30 µg of each fraction were analyzed by Western blotting with anti-cytochrome c antibody. Protein loading was confirmed by β-tubulin Western blot analysis. Expression of ARF was checked by anti-p19ARF antibody. B, mitochondrial membrane potential. To monitor mitochondrial membrane potential (MTR, top panels), cells were loaded with 100 nM MitoTracker® Red CMXRos (Molecular Probes) in culture medium at 37 °C for 15 min 12 h after starvation. Bottom panels (DIC) are differential interference contrast images.

A large percentage of apoptotic nuclei in ARF-infected cells (Fig. 5B). In contrast, pro-caspase-8 cleavage and accumulation of p18, the active form of caspase-8, were not observed in ARF virus-infected cells at all (data not shown), consistent with our data that ARF modulates Bcl-2 family proteins in mitochondria. The modulation of Bcl-2 family proteins induces cytochrome c release and affects mitochondrial membrane potentials (Figs. 1–4). The cleaved form of caspase-8 was faintly detectable in ARF-expressing cells 10 h after serum starvation and increased (Fig. 5C), suggesting that apoptotic signals brought about by ARF activate an effector caspase (caspase-3) via a mitochondrial pathway.

Fig. 5. Caspase-9 and caspase-3 are activated by ARF. A, cells were infected with high-titer recombinant control virus (mock) and ARF virus (ARF) and then cultured with 10% (v/v) serum media for 24 h with (ZVAD) or without a general caspase inhibitor, ZVAD-fmk, at 100 µM. Starvation for 24 h was performed at the same concentration of ZVAD-fmk before serum depletion. Apoptotic nuclei were assayed by staining with Hoechst 33258. Percentages of apoptotic nuclei were scored as follows: f, fragmented nuclei; c, condensed nuclei; and t, total, sum of f + c. Results are the mean ± S.D. of four to six independent experiments. B and C, processing of pro-caspase-9 (B) and pro-caspase-3 (C) induced by ARF. Cells were infected with a control virus (mock) or ARF virus (ARF), starved, collected at the indicated time points, and subjected to SDS-PAGE/Western blot analysis. Processing of pro-caspase-9 was detected by the presence of 35/37-kDa cleaved forms. Anti-caspase-3 mouse monoclonal antibody (clone 46) recognizes the 32-kDa pro-caspase-3 and the 20-kDa cleaved form.
Bax by p53-KH215 alone seems to be consistent with previous reports, which describe p53-dependent Bax induction (34). After serum starvation, ARF overexpression caused Bax/Bim EL up-regulation and Bcl-2 down-regulation in mitochondrial fractions (Fig. 6D). Unexpectedly, co-expression of ARF/p53-KH215 up-modulated mitochondrial Bim EL/L and Bax, suggesting the possibility that the dominant-negative p53 inhibited the p53-dependent signals normally caused by ARF and compensatorily induced p53-independent Bim/Bax-inducing signals. It is also noteworthy that p53-KH215 recovered Bcl-2 amounts in mitochondrial fractions of ARF-expressing cells (Fig. 6D). In total cell lysates, Bcl-2 amounts were slightly lower in ARF-expressing cells, and Bax/Bim amounts were slightly higher in ARF-expressing cells. Because ARF/p53-KH215 was located mainly in the nuclear fraction (pellet), modification of Bcl-2 family proteins in mitochondrial fractions seems to be a downstream effect of an ARF/p53 pathway.

**DISCUSSION**

**ARF Disrupts Mitochondria Function and Triggers Apoptosis**—The Bcl-2 protein family consists of proapoptotic and antiapoptotic members that regulate the life and death of a cell by controlling the release of mitochondrial apoptogenic factors such as cytochrome c (35) and apoptosis-inducing factor (36). Proapoptotic Bcl-2 family proteins consist of “BH3-only proteins” and “BH1 through 3 proteins.” The proapoptotic protein Bax redistributes in and acts on mitochondria to induce cell death (28). Many cellular stresses, e.g. chemicals (38), growth factor starvation, serum starvation in human vessel cells (39), and interleukin 2 deprivation in T cells (40), induce Bax apoptosis induction. A novel BH3-only subfamily member, Bim, was isolated independently by two groups (41, 42) who exploited its ability to bind Bcl-2 or Mcl-1. Alternative splicing products, Bim EL, Bim L, and Bim S, were identified and also function as death inducers (43).

In recent years, it has been well established that antiapoptotic Bcl-2 family proteins prevent most forms of apoptotic cell death induced by various stimuli, such as growth factor starvation, chemotherapeutic agents, and heat shock. Both Bcl-2 and Bcl-xL overexpression have been shown to prevent all mitochondrial apoptotic changes, including cytochrome c release and loss of membrane potential (44, 45). Several proapoptotic stresses cause mitochondrial dysfunction resulting in translocation of cytochrome c from the mitochondria to the cytosol (46–48). Cytochrome c directly activates caspase cas-
ARF-induced Apoptosis in the Absence of Growth Factors

cades by binding to a cytoplasmic protein, Apaf-1, via the C-terminal WD-40 repeat domain in the presence of ATP or dATP, resulting in an oligomer complex that activates caspase-9. Once activated, caspase-9 can initiate an apoptotic cascade involving the downstream executioners caspase-3, -6, and -7 (29).

ARF expression in serum-starved cells modulated the amounts of Bcl-2 and Bax/Bim in mitochondrial fractions (Fig. 2) resulting in cytochrome c translocation from the mitochondria to the cytosol (Fig. 4A) and decreased mitochondrial membrane potential (Fig. 4B). Active forms of caspase-9 appeared after cytochrome c release (Fig. 5B). After caspase-9 activation, efficient processing of caspase-3, an effector caspase activated by cleaved caspase-9 (49), occurred in ARF-overexpressing cells (Fig. 5C), suggesting an ARF-induced apoptotic pathway dependent on both caspase-9 and caspase-3. In agreement with these results, a general caspase inhibitor, zVAD-fmk, protected the cells from ARF-induced apoptosis (Fig. 5A). Another report has shown that caspase-9-null mouse embryo fibroblasts and Apaf-1-null cells are resistant to p53-dependent stresses (50), an observation that supports the idea that cytotoxic stress from the ARF-p53 pathway is involved in mitochondrial dysfunction resulting in caspase-9 activation. These results lead us to believe that activation of the caspase cascade via the mitochondrial apoptotic pathway is one of the essential mechanisms for ARF-induced apoptosis.

The Role of the ARF-p53 Pathway in Regulation of Bcl-2 Family Proteins—Bcl-2 is a product of the proto-oncogene BCL-2, which was discovered at the chromosomal breakpoint of t(14:18)-bearing human B-cell lymphoma. Antiapoptotic Bcl-2 and Bcl-xl prevent all mitochondrial apoptotic changes including cytochrome c release and mitochondrial membrane potential loss (44, 45). In our experiments, Bcl-2 expression was down-modulated by ARF overexpression after serum starvation in mitochondrial fractions of p53 wild-type, ARF/p16-null fibroblasts (Fig. 2). Furthermore, Bcl-2 and Bcl-xl co-expression effectively suppressed ARF-induced apoptosis in serum-free conditions. These results indicate that antiapoptotic Bcl-2 family proteins, particularly Bcl-2, are key antiapoptotic molecules in ARF-induced apoptosis.

There are several reports that have studied the modulation of Bcl-2 family protein expression by p53 and the downstream molecules of p53 (51, 52). In these studies, some have demonstrated that Bcl-2 gene expression is repressed in the presence of wild-type p53 at the transcriptional level (34). In the case of p53-dependent apoptosis, Bcl-2 expression was found to be down-regulated in human breast cancer cells (53) and lymphoma cells (54).

These results prompted us to use dominant-negative p53 to analyze the role of p53 in ARF-induced apoptosis and ARF-dependent Bcl-2 family protein modulation. A mutant mouse p53, p53-KH215, was shown to accumulate to the same degree as wild-type p53 in COS7 cells after transfection of expression plasmids (22). Co-expression of p53-KH215 with ARF considerably repressed apoptotic nuclear changes (Fig. 6A), internucleosomal DNA fragmentation (Fig. 6B), and sub-G1 fraction in FACS analysis (Fig. 6C). In concordance, co-expression of p53-KH215 with ARF recovered the amounts of Bcl-2 in mitochondrial fractions (Fig. 6D), confirming that Bcl-2 down-regulation in ARF-induced apoptosis is essential and occurs via a p53 pathway.

On the other hand, co-expression of p53-KH215 with ARF further up-regulated Bim EL/L and Bax in mitochondrial fractions after serum starvation (Fig. 6D). These results may indicate that the increase of Bim EL/L and Bax in mitochondrial fractions of ARF-expressing cells is not dependent on p53 but rather on a downstream pathway independent of ARF, which is compensatorily induced by inhibition of the ARF-p53 pathway. In agreement with our results, lines of evidence have indicated that ARF-MDM2-p53 functions through a complex network rather than a simple linear pathway. Mice engineered to overexpress a c-Myc transgene develop B-lymphoid malignancies, with a majority of the resulting tumors arising from ARF deletion, p53 mutation, or MDM2 overexpression. Several tumors that lacked ARF or p53 function overexpressed MDM2, inviting argument against a simple epistatic relationship between the genes (55). Moreover, ARF/MDM2/p53 triple knockout mice develop multiple tumors at a frequency greater than those observed in animals lacking both p53 and MDM2 or p53 alone, suggesting that ARF can act independently of the MDM2-p53 axis in tumor surveillance (37). Another explanation of Bax/Bim up-regulation is that p53-KH215 may partially inhibit the wild-type p53 function to suppress Bcl-2 but may not inhibit the function to induce proapoptotic Bcl-2 family proteins. Furthermore, we cannot deny the possibility that p53-KH215 may affect other mitochondrial Bcl-2 family proteins in ARF-induced cell death.

In conclusion, this article has demonstrated the molecular mechanism of ARF-induced apoptosis in the absence of growth factors. ARF expression modulated Bcl-2 family proteins in mitochondrial fractions under serum starvation and induced mitochondrial dysfunction, resulting in activation of downstream caspases of mitochondrial proapoptotic signals. In mitochondrial fractions, loss of antiapoptotic Bcl-2 expression induced by ARF is caused via a p53-dependent pathway. Up-regulation of proapoptotic Bax/Bim expression by ARF seems to rely on a downstream ARF pathway that is p53-independent, as shown in experiments with a dominant-negative mouse p53. This is the first report that has studied the molecular mechanism of ARF-induced apoptosis and indicates an ARF-activated pathway for cell death independent of p53. Additional studies to analyze the molecular mechanism of MDM2-p53-independent ARF-induced cell death are of great interest for understanding self-defense mechanisms against oncogenic stress.

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ARF Tumor Suppressor Induces Mitochondria-dependent Apoptosis by Modulation of Mitochondrial Bcl-2 Family Proteins
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