The Alternative Reading Frame Tumor Suppressor Inhibits Growth through p21-dependent and p21-independent Pathways

Modestos Modestou, Valerie Puig-Antich, Chandrashekar Korgaonkar, Alex Eapen, and Dawn E. Quelle

Department of Pharmacology and the Molecular Biology Graduate Program, The University of Iowa, College of Medicine, Iowa City, Iowa 52242

ABSTRACT

The alternative reading frame (ARF) tumor suppressor mediates growth arrest or apoptosis through activation of the p53 tumor suppressor. A prevailing concept is that ARF uses p21Cip1/Waf1, a p35-responsive gene and cyclin-dependent kinase (Cdk) inhibitor, to block cell cycle progression. Using p21 nullizygous cells, we demonstrate that p21 is nonessential for the antiproliferative activity of ARF and p53, although it likely governs the arrest through Cdk inactivation when present. ARF overexpression in p21-positive and p21-negative mouse embryo fibroblasts (MEFs), but not in primary cells lacking p53, induced a biphasic (G1 and G2) cell cycle arrest. The ARF-induced growth arrest, regardless of p21 status, coincided with activation of p53 and accumulation of hypophosphorylated retinoblastoma protein (retinoblastoma protein). In ARF-arrested p21-positive cells, the presence of growth-inhibitory retinoblastoma protein correlated with an absence of Cdk2-dependent kinase activity, an increase in p21 association with inactive Cdks, and a lack of cyclin A expression. In contrast, p21+/− mouse embryo fibroblasts were arrested by ARF despite containing elevated levels of cyclin A protein and highly active Cdk2-dependent kinases. These findings provide evidence that ARF can block growth through a p21-independent pathway(s) that overrides Cdk2 activation.

INTRODUCTION

The first and second most frequently inactivated genes in neoplastic transformation are p53 and INK4a/ARF (1). p53 serves to maintain genomic stability in response to DNA damage, oncogenic insults, and other cellular stresses (2). Normally, the p53 protein is maintained at low levels in proliferating cells, but it rapidly accumulates in response to cellular stress and transactivates genes that trigger growth arrest or apoptosis. Failure to activate p53-dependent checkpoints limits the repair and removal of damaged cells from an organism, and increases the chances that a cell will become cancerous. ARF,3 a product of the INK4a/ARF locus, prevents cellular transformation and immortalization by activating p53 in response to activated oncogenes and during replicative senescence (1). ARF induces p53 up-regulation by neutralizing the effects of Mdm2, a transcriptional target of p53 that antagonizes its function. Although ARF has no activity in p53-negative cells, its ability to suppress the growth of cells lacking both p53 and Mdm2 indicates that it can function independently of p53 when Mdm2 is also eliminated (3).

The outcomes of ARF-induced p53 activation are cell death or G1 and G2 phase growth arrest (1). In primary fibroblasts, apoptosis is promoted by ARF in response to hyperproliferative signals conveyed by oncoproteins such as c-Myc (4) and adenovirus E1A (5). Conversely, activation of ARF in fibroblasts by oncogenic Ras (6, 7) and v-Abl (8) results in an irreversible cell cycle arrest, termed cellular senescence, as does enforced expression of ARF alone (9, 10). Significantly, p53 activation by any of these stimuli is either abolished or severely impaired in ARF-null MEFs, and cells lacking p53 are refractory to the antiproliferative effects of ARF (4, 5, 8, 9, 11). By the same token, activated oncogenes promote unrestrained cell growth by selectively dismantling the ARF-p53 pathway (1). The importance of ARF and p53 in vivo has been directly demonstrated by the high frequency of tumor development in mice lacking either protein (11–13).

Activation of p53 by ARF is associated with up-regulation of the p53-responsive gene, p21Cip1/Waf1 (9, 11). As a potent Cdk inhibitor, p21 is commonly perceived to be the major effector of p53-mediated cell cycle arrest (2, 14). Mammalian cell growth is governed by a series of Cdks whose sequential activation in G1 to S phase (from cyclin D/Cdk4 to cyclin E/Cdk2 and cyclin A/Cdk2) and G2–M (cyclin B/Cdk1 (Cdc2)) promotes orderly cell cycle progression (15). p21 can inhibit all of these kinases in vitro (16), and its association with inactivated cyclin/Cdk complexes coincides with its ability to invoke G1 and G2–M growth arrest (16–18). In ARF-arrested fibroblasts, Cdk1−, Cdk2−, and Cdk4-dependent kinases are all inactivated. Because ARF does not directly interact with cyclins or Cdks (19, 20), a prevailing concept is that p21 mediates ARF-induced growth arrest. A functional link among ARF, p53, and p21 is further implied by observations that each protein can provoke premature senescence (9, 10, 21, 22).

Other work, however, has shown that p53-mediated growth inhibition is not strictly dependent on p21. For instance, the c-Abl tyrosine kinase induces G1 phase arrest in p21-negative MEFs, but not p53−− fibroblasts, indicating that it functions in a p53-dependent, p21-independent manner to inhibit growth (23). Also, unlike ARF or p53-deficient mice and MEFs (11, 24), p21-null animals remain tumor-free throughout their lifetime, and their MEFs retain senescence capability (25, 26). Thus, factors other than p21 are likely required for the tumor suppressor functions of p53 and ARF. Here, we suggest that ARF normally induces a G1 and G2 phase growth arrest through p21-dependent inactivation of Cdks, but in the absence of p21, an alternate pathway(s) becomes activated. These data show that p21 is not required for ARF- and p53-mediated growth arrest and indicate that multiple downstream effectors exist that mediate the growth suppressive functions of ARF.

MATERIALS AND METHODS

Cell Culture Conditions and Cell Cycle Analyses. MEFs lacking p21, p53, both p21 and p27, or ARF (kindly provided by Phil Leder (Harvard University, Boston, MA), Tyler Jacks (Massachusetts Institute of Technology, Boston, MA), Jim Roberts (Fred Hutchinson Cancer Research Center, Seattle, WA), and Chuck Sherr and Martine Roussel (St. Jude Children’s Research Hospital, Memphis, TN, respectively); human kidney 293T cells; and NIH 3T3 fibroblasts overexpressing cyclin D1 (27) were maintained in complete medium (DMEM containing 10% fetal bovine serum, 2 mM glutamine, and 100 μg/ml of penicillin and streptomycin). Complete medium was supplemented with 55 μM 2-mercaptoethanol and 0.1 mM nonessential amino acids for growth of ARF−− MEFs. Leptomycin B, a generous gift of Minoru Yoshida (The University of Tokyo, Tokyo, Japan), was used at 5 ng per ml in...
complete medium to treat exponentially growing cells for 12 or 24 h. DNA content was measured by flow cytometry (Becton Dickinson FACScan) after staining cells with propidium iodide (19).

Retroviruses encoding HA-tagged mouse ARF and CD8 or CD8 alone were produced by transient transfection of 293T cells with an ecotropic helper virus DNA plus bicistronic pSRα-murine sarcoma virus-tkCD8 vector plasmids containing HA-ARF or no insert (28). Infections of low-density cell cultures were performed as described previously (28), except that cells (1 × 10^6 cells per 150-mm diameter culture dish) were treated for 6 h with 7 ml of virus (supplemented with 8 μg/ml polybrene), followed by the addition of 18 ml of fresh complete medium. Cells were analyzed by dual-color flow cytometry 60–72 h postinfection to determine the DNA content of CD8-positive cells (28).

Western Blot Analyses. Frozen cell pellets were lysed at 1 × 10^6 cells/ml on ice for 1 h in NP40 buffer [50 mM Tris (pH 7.5), 120 mM NaCl, 1 mM EDTA, and 0.5% NP40] supplemented with 0.1 mM sodium vanadate, 1 mM sodium fluoride, 5 μg/ml leupeptin, and 30 μM phenylmethylsulfonyl fluoride. For analyses of pRb, cells were lysed in 25 mM Tris (pH 7.5), 192 mM glycine, and 3.5 mM SDS. After vortexing and sonication (two 5-s pulses), lysates were clarified by centrifugation at 4°C. Equivalent amounts of protein were separated on denaturing gels, electroblotted onto PVDF membranes (Millipore), and detected by enhanced chemiluminescence (Amersham). Antibodies were purchased from Santa Cruz Biotechnologies (Cyclin E, Cdk1, Cdk2, Cdk4, and p27), Calbiochem (Cyclin A, Cdk1, and p21), or New England Biolabs and PharMingen (pRb). Antisera to ARF (19) and cyclin D1 (27) were described previously. p53 and Mdm2 were detected using pAb 421 and 2A10 hybridoma supernatants, respectively (kindly provided by Gerry Gambetti [St. Jude Children’s Research Hospital] and Arnold Levine [Rockefeller University, New York, NY]).

Kinase Assays. NP40 lysates (200 μg of protein) were subjected to immunoprecipitation with antibodies to Cdk1, Cdk2, or Cyclin A for 2 h at 4°C. Immunocomplexes were assayed for histone H1 kinase activity as described previously (29), except that proteins were transferred to nitrocellulose after SDS-PAGE to allow for Western blotting. Quantification of [γ-32P]ATP incorporation into histone H1 was performed on a PhosphorImager (Molecular Dynamics).

RESULTS

The Cdk Inhibitor, p21, Is Not Required for ARF- and p53-mediated Growth Suppression. We tested the role of p21 in ARF-mediated growth arrest by examining the growth suppressive activity of ARF in MEFs derived from p21 knockout mice. Cells were infected with bicistronic retroviruses encoding ARF and the CD8 cell surface marker, or control vector viruses encoding only CD8. Three days postinfection the cell cycle distributions of successfully infected (CD8-positive) cells were determined by dual-color flow cytometry (Fig. 1A; Table 1). Fibroblasts expressing the vector virus were unaffected and proliferated asynchronously. As anticipated, ARF had no effect in cells lacking p53, whereas ARF−/− MEFs and NIH 3T3 fibroblasts (each bearing wild-type p53 and p21 proteins) became arrested in G1 and G2 with nearly complete loss of S phase cells (9, 11, 19). An NIH 3T3 derivative expressing high levels of cyclin D1 was used in these studies because it undergoes a more dramatic G2 arrest in response to ARF than parental cells do (19, 28). Notably, ARF elicited a biphasic growth arrest in p21−/− MEFs nearly identical to that seen in the p21+/+ fibroblasts, except that a greater percentage of cells accumulated in G2 phase (Table 1). Likewise, MEFs lacking both p21 and the related Cdk inhibitor, p27, were blocked in G1 and G2 by ARF, as recently observed by Groth et al. (30). The ability of ARF to suppress growth in a p21-independent manner was reproducibly demonstrated by the reduction in S phase (Fig. 1B) and cell number (Fig. 1C) in p21-negative cell types compared with vector-infected controls. These data show that p21 is not required for ARF-mediated growth inhibition.

The lack of S phase reduction and minimal decrease in cell number in p53−/− MEFs expressing exogenous ARF supports the view that ARF-induced growth inhibition is largely mediated through p53 (1). Because ARF inhibits the growth of p21-negative cells, these results imply that p53-mediated growth arrest can occur independently of p21. To confirm that notion in a system removed from the effects of ARF, each cell population was treated with or without 10 mM leptomycin B for 12 to 24 h and their DNA content examined by flow cytometry (Fig. 2). Leptomycin B is an antibiotic that specifically inhibits CRM1-mediated nuclear export of proteins containing HIV1 Rev type nuclear export signals, such as Mdm2 (31, 32). Treatment of cells with leptomycin B inhibits Mdm2-mediated export and proteosomal degradation of p53, leading to accumulation of p53 in the nucleus and induction of p53-dependent transcriptional activity (33, 34). Thus, leptomycin B would be expected to block the growth of cells expressing wild-type p53. We found that NIH 3T3, ARF−/−, and p21−/− fibroblasts, all of which are p53-positive, became ar-

![Image](image-url)
ARF INDUCES p21-INDEPENDENT GROWTH ARREST

Table 1 Comparison of cell cycle distributions for ARF-arrested cells

<table>
<thead>
<tr>
<th>Cell type and infection</th>
<th>G1</th>
<th>S phase</th>
<th>G2/M</th>
</tr>
</thead>
<tbody>
<tr>
<td>NIH 3T3-D1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vector</td>
<td>39 ± 3.6</td>
<td>28 ± 2.9</td>
<td>33 ± 3.7</td>
</tr>
<tr>
<td>ARF</td>
<td>61 ± 2.1</td>
<td>5 ± 1.7</td>
<td>35 ± 0.7</td>
</tr>
<tr>
<td>ARF−/− MEF</td>
<td>48 ± 4.8</td>
<td>24 ± 5.1</td>
<td>28 ± 7.5</td>
</tr>
<tr>
<td>p21−/− MEF</td>
<td>67 ± 7</td>
<td>7 ± 2</td>
<td>26 ± 6.7</td>
</tr>
<tr>
<td>Vector</td>
<td>31 ± 1.7</td>
<td>30 ± 3.3</td>
<td>39 ± 4.6</td>
</tr>
<tr>
<td>ARF</td>
<td>47 ± 5.3</td>
<td>7 ± 0.8</td>
<td>46 ± 5.9</td>
</tr>
</tbody>
</table>

The ability of ARF to block cell cycle progression is most likely attributable to alterations in the activity of one or more cell cycle regulators. ARF-arrested NIH 3T3 cells contain inactive Cdk1, Cdk2, and Cdk4 kinases, yet ARF cannot directly inhibit Cdkks (19, 20). Thus, the lack of Cdk activity could result from reduced cyclin or Cdk expression, the binding to Cdk inhibitors (such as p21), or the inability of the cyclin and Cdk subunits to form complexes. We first examined the expression patterns of various cell cycle regulators in vector- or ARF-infected cells by immunoblotting (Fig. 3). Most showed little to no change in expression level in response to ARF, including cyclin D1, cyclin E, Cdk2, Cdk4, and the p21-related Cdk inhibitor, p27. The lack of p27 up-regulation suggests that ARF-induced growth arrest in p21-negative cells is not attributable to compensating Cdk inactivation by another p21 family member, consistent with the ability of ARF to inhibit the growth of p21/p27-null MEFs (Fig. 1). By comparison, ARF caused reduced expression of the G2-M-specific kinase, Cdk1, in both NIH 3T3 and p21−/− MEFs (Lanes 1–4), consistent with the inability of ARF-arrested cells to enter mitosis (19). p53-null MEFs continued to proliferate in the presence of exogenous ARF and no reduction in Cdk1 expression was observed (Lanes 5 and 6).

A major difference between ARF-arrested NIH 3T3 and p21−/− fibroblasts was the expression pattern of cyclin A. In NIH 3T3 cells, ARF overexpression was associated with dramatically reduced levels of cyclin A (Lane 2 versus 1), a result also observed by Kurokawa et al. (20), whereas cyclin A continued to be expressed in p21-negative MEFs arrested by ARF (Lane 4 versus 3). To assess the activity of cyclin A in those cells, immune complex kinase assays were performed (Fig. 4A). Cyclin A-associated histone H1 kinase activity was abolished in ARF-arrested NIH 3T3 cells, in agreement with their low level of cyclin A expression and our own previous findings (19). In contrast, cyclin A-dependent kinases were active in ARF-arrested p21−/− MEFs. Quantification of the band intensities revealed a 35% decrease in kinase activity in those cells compared with proliferating controls, corresponding to the level of cyclin A expressed (Fig. 3) and the amount of its catalytic partner, Cdk2, in the complex. In p53−/− ARF (11, 19). In NIH 3T3 fibroblasts, ARF stimulated the accumulation of p53, Mdm2, and p21 proteins (Lane 2 for each blot). These results were anticipated and indicative of p53 activation. Unexpectedly, p21−/− MEFs expressed high basal levels of p53 protein that were not enhanced by treatment with ARF (Lane 4 versus 3). However, Mdm2 was up-regulated, which strongly suggests that p53 was activated in those cells. As transcriptional targets of p53, Mdm2 and p21 proteins were either weakly expressed or not detected, respectively, in p53−/− MEFs (Lanes 5 and 6).

Distinct Molecular Phenotypes of ARF-arrested Cells Depending on p21 Status. ARF suppresses proliferation by binding to Mdm2 and preventing its down-regulation of p53, resulting in accumulation of p53 and up-regulation of its target genes, including Mdm2 and p21 (1). To better characterize the nature of the ARF-mediated growth inhibition in p21−/− cells, changes in the expression of these proteins after ARF infection were examined by Western blot analysis (Fig. 3). Because of the large scale of each experiment, repeated analyses were limited to cells representing the three different genotypes (i.e., p53−/−, p21−/− MEFs, and p53−/− MEFs). However, identical results were obtained in NIH 3T3 fibroblasts and primary ARF−/− MEFs for all experiments described (data not shown). Importantly, although the percentage of retrovirally transduced cells ranged from 65 to 100% in individual experiments, biochemical analyses were performed only on populations in which >90% were successfully infected, as determined by expression of CD8.

As shown in the Fig. 3A, HA-tagged ARF protein was introduced at high levels in each cell type infected with ARF retroviruses (Lanes 2, 4, and 6). No endogenous ARF was detected in vector-infected NIH 3T3 cells that lack the INK4a/ARF gene, nor in p21-null MEFs, which (like most p53-positive cells) express very low levels of the protein (19). In contrast, p53−/− cells express high levels of endogenous ARF. In NIH 3T3 fibroblasts, ARF stimulated the accumulation of p53, Mdm2, and p21 proteins (Lane 2 for each blot). These results were anticipated and indicative of p53 activation. Unexpectedly, p21−/− MEFs expressed high basal levels of p53 protein that were not enhanced by treatment with ARF (Lane 4 versus 3). However, Mdm2 was up-regulated, which strongly suggests that p53 was activated in those cells. As transcriptional targets of p53, Mdm2 and p21 proteins were either weakly expressed or not detected, respectively, in p53−/− MEFs (Lanes 5 and 6).
MEFs, cyclin A expression and association with Cdk2 was unchanged by ARF, although ARF did cause a detectable reduction in the associated kinase activity. Immunoblotting of the kinase reactions revealed that none of the active complexes contained p21, and only low levels of p21 associated with cyclin A in ARF-arrested NIH 3T3 fibroblasts (data not shown), reflecting the near absence of cyclin A expression in those cells.

Similar analyses of Cdk2-associated kinases were performed (Fig. 4B), and the results matched those obtained for cyclin A. Cdk2-dependent kinases (which includes cyclin E/Cdk2 and cyclin A/Cdk2 complexes) were inactive in ARF-arrested NIH 3T3 cells but remained active in ARF-arrested p21−/− cells. Two forms of Cdk2 were observed in these IPs, and the presence of the active form (faster-migrating because of activating phosphorylation) correlated with measurable Cdk2 kinase activity. By comparison, phosphorylated Cdk2 was not associated with inactive immune complexes from NIH 3T3 arrested cells. Reprobing the kinase blot for p21 showed that high levels coprecipitated with active Cdk2 from control NIH 3T3 cells, whereas an approximate 2-fold increase in associated p21 coincided with inactivation of those complexes. No p27 was detected in cyclin A or Cdk2 immune complexes, regardless of treatment condition (data not shown).

Given that cyclin A/Cdk2 activity is rate limiting and is required for S phase entry (35, 36), the presence of active cyclin A/Cdk2 kinases in growth arrested p21-null cells was unexpected. Therefore, we examined the phosphorylation status of pRb, a physiological substrate of Cdk4- and Cdk2-dependent kinases, in growth arrested NIH 3T3 cells (Fig. 4C; Ref. 15). As anticipated, hypophosphorylated pRb accumulated in ARF-arrested p21-positive NIH 3T3 cells (Fig. 4C, upper panel), and phosphorylation of pRb at a Cdk2-specific site, Ser-811, was severely impaired (Fig. 4C, lower panel; Refs. 9, 20). By comparison, proliferating vector-infected cells expressed hyperphosphorylated pRb. In p53−/− MEFs, lower overall levels of pRb expression and hyperphosphorylation were observed, but no difference in pRb phosphorylation was induced by ARF. Interestingly, p21−/− MEFs, arrested by ARF, behaved like p21+/+ cells, displaying clearly reduced levels of hyperphosphorylated pRb.

To better understand the basis of the G2 phase cell cycle arrest elicited by ARF, we examined Cdk1-dependent kinase activity in the different cell types (Fig. 5). The reduction of Cdk1 expression in ARF-infected NIH 3T3 cells correlated with near complete loss of Cdk1-associated histone H1 kinase activity. Very little Cdk1 protein was detected in IPs from these cells, yet equivalent amounts of p21 were present in the complexes from both vector and ARF-treated samples. Thus, a significantly higher amount of p21 was bound per Cdk1 molecule in ARF-arrested cells, and this may contribute to its inactivation. In comparable assays of p21-null MEFs, ARF overexpression led to a more modest level of Cdk1 inhibition, but the baseline of inhibited kinase activity was similar in both NIH 3T3 and p21-null cells. The modest reduction in kinase activity in ARF-arrested p21−/− MEFs results from low endogenous levels of Cdk1 activity in the growing, vector-infected population, which is most likely a consequence of impaired cyclin B1 accumulation in the nuclei of those cells (18). No difference in Cdk1 expression or kinase activity was induced by ARF in p53−/− MEFs.

**DISCUSSION**

Our studies indicate that both p21-dependent and p21-independent pathways can be activated by ARF to prevent growth. ARF-mediated growth arrest in cells bearing wild-type p53 and p21 (NIH 3T3 cells and ARF-null MEFs) correlated with p21 up-regulation, and increased association of p21 with inactivated Cdk1, Cdk2 and Cdk4 complexes (data herein and Refs. 19, 20). These observations are consistent with a role for p21 in growth inhibition by ARF. However, we found that ARF inhibited the growth of p21-negative cells, which suggests that, in the absence of p21, another mechanism(s) is activated by ARF to inhibit cell cycle progression. Recent reports, showing that oncogenic Ras, an activator of ARF, can inhibit the growth of p21-null and p21/p27-null MEFs, support such a conclusion (26, 30). In our study, the p21-independent pathway of ARF-mediated growth arrest has two notable features. First, the arrest is biphasic with one-half of cells blocked in G1 and the other half in G2; and second, it coincides with accumulation of hypophosphorylated pRb despite expression of cyclin A and activated Cdk2 kinases.

The biphasic nature of the ARF-induced p21-independent growth arrest is significant for its implications regarding G1 checkpoint control and senescence. Nearly one-half of p21-null cells were arrested by ARF with 2n DNA content, a percentage that is slightly reduced compared with p21-positive cells but that nonetheless represents a substantial G1 population. Because ARF functions in a p53-dependent manner under normal circumstances (1), those results suggest that MEFs lacking p21 are not defective for p53-mediated G1 checkpoint control. The existence of p53-dependent, p21-independent
Mechanisms of growth arrest is also supported by our observation that leptomycin B blocks the cell cycle in p53-positive p21−/− cells, but not in MEFs lacking p53. Likewise, Yuan et al. (23) reported that c-Abl induces p53-mediated G1 arrest in cells lacking p21. By comparison, other studies showed that MEFs and human cancer cells lacking p21 are either severely or completely impaired in their G1 checkpoint response to DNA damage (25, 37, 38). Such findings imply that ARF dictates a different antiproliferative signal through p53 than does DNA damage. Although one report places DNA damage and ARF in a common pathway upstream of p53 (39), up-regulation of ARF by oncogenes or enforced overexpression differs from the effects of DNA damaging agents in that it does not cause overt cell injury and it does not activate p53 via serine-15 phosphorylation (5).

Both p21-positive and p21-negative cells arrest in G2 phase in response to ARF. As one would predict, this correlated with reduced expression and activity of the G2-M phase kinase, Cdk1. Recent studies show that p21 may be dispensable for initiation of a G2 checkpoint but required for its maintenance (40–43). Specifically, p21−/− MEFs and HCT116 colon cancer cells exposed to anticancer agents exhibited a transient G2 arrest followed by endoreduplication and apoptosis. Polyplody and cell death were maximal 48 to 60 h after irradiation or drug treatment, whereas similarly treated p21+/+ cells remained growth arrested over a 4-day period. The basis of the sustained growth arrest by p21 may depend on its ability to inhibit Cdk2 and prevent aberrant DNA replication, because Cdk2-dependent kinase activity is high in endoreduplicating cells (44). We observed cyclin A- and Cdk2-associated kinase activity in ARF-arrested p21-null MEFs, whereas all Cdk activity was abolished in p21-positive cells. This raises the possibility that p21−/− MEFs, unlike normal primary fibroblasts (9, 10), cannot permanently exit the cell cycle and undergo senescence in response to ARF. In that regard, it is noteworthy that no significant difference in ploidy or cell death has been apparent in p21-null cells that expressed vector or ARF retroviruses for 4 days. Rather, both ARF−/− and p21−/− MEFs expressing exogenous ARF became remarkably larger and flatter in appearance, characteristic of the senescent phenotype (data not shown). That is in keeping with the observation that the loss of p21 does not impair Ras-induced senescence (26).

A key distinction between p21-negative and p21-positive cells that are arrested by ARF is the presence of cyclin A protein and activated cyclin A/Cdk2 kinases in p21−/− MEFs. Normally, cyclin A mRNA and protein expression does not begin in fibroblasts until the end of G1 phase, when its transcriptional activator, E2F, becomes active because of pRb hyperphosphorylation (35, 45). As such, the cyclin A expression data initially suggested a late G1 or early S phase arrest in cells lacking p21 versus an earlier G1 block in p21-positive cells. However, the detection of hypophosphorylated pRb in both p21−/− and p21+/+ cells expressing ARF makes it unclear whether the timing of the 2n arrest in the two cell types is different. Conceptually, the expression of cyclin A is at odds with the accumulation of hypophosphorylated pRb. Such a situation might arise if pRb-related pocket proteins, p107 and p130, were phosphorylated and they released other E2Fs that pushed cell progression further toward G2-S phase. That would be consistent with the presence of active Cdk2-dependent kinases in these cells, because Cdk2 can phosphorylate all of the pocket proteins, and it normally drives S phase entry (15). Alternatively, cyclin A expression may be E2F-independent under these conditions. In either case, the lack of Cdk2-mediated pRb phosphorylation is unresolved. It is possible that the status of pRb reflects: (a) a lack of prior Cdk4-dependent pRb phosphorylation that normally enables Cdk2 to act on pRb (45, 46); (b) a failure of the Cdk2 kinases to reach a critical threshold level (because its activity is partially reduced in p21-null arrested cells), which is required to phosphorylate pRb and other physiological substrates; and (c) a mislocalization of the kinase to the cytoplasm in p21-negative cells, as reported for cyclin B1 (18).

The importance of pRb in ARF-mediated growth suppression has not yet been established. One report suggests that ARF can inhibit growth through pRb in cells lacking functional p53 (47). Others recently showed that ARF can function independently of pRb, at least in MEFs lacking both p53 and Mdm2, because overexpression of the E7 oncprotein had no effect on ARF’s antiproliferative activity (3). However, all previous work in less manipulated systems suggests that ARF functions upstream of p53 and requires its activation to elicit growth arrest, and, in those studies, the role of pRb is implied (1, 9, 11, 48). Specifically, it is predicted that ARF ultimately inhibits growth through pRb, based on the up-regulation of p21, inhibition of Cdk4, and absence of hypophosphorylated pRb in wild-type ARF-arrested cells (9, 19, 20). Our finding that hypophosphorylated pRb also accumulates in ARF-arrested p21−/− cells suggests that growth suppression by pRb might be required for the antiproliferative activity of ARF in p53-positive cells. Still, it remains to be proven in the p21-null background whether pRb phosphorylation status is a cause or consequence of the growth arrest.

The work presented here prompts the question, what is the molecular basis of p21-independent ARF-induced growth suppression? Although pRb may ultimately be involved, the identity of a more proximal regulator(s) of the arrest remains unknown. For a number of reasons, it seems most likely that other p53 targets capable of blocking the cell cycle may play a key role. First, p53 appears to be activated in ARF-arrested p21−/− MEFs, based on the up-regulation of Mdm2 and another p53-responsive gene, cyclin G1 (data not shown). Second, the arrest phenotype induced by ARF in p21-null MEFs differs from that seen in p53/Mdm2-null MEFs, which suggests that distinct regulators mediate arrest in the two backgrounds. Specifically, unlike the growth inhibition in cells lacking p53 and Mdm2 (3), the kinetics of ARF-induced arrest in p21-negative MEFs was not significantly delayed compared with wild-type cells, and cells did not preferentially accumulate at the G1-S phase boundary. Together, the data presented here and in the literature suggest that ARF can activate multiple antiproliferative pathways downstream of p53, as well as independent of p53. Whereas the mediators of the alternate arrest pathways remain to be identified, these findings speak to the resourcefulness and potency of ARF as a tumor suppressor.

ACKNOWLEDGMENTS

We thank Phil Leder, Tyler Jacks, Jim Roberts, Chuck Sherr, Martine Roussel, Minoru Yoshida, Gerry Zambetti, and Arnold Levine for their generous provision of reagents; Justin Fishbaugh and Gene Hess for excellent assistance with flow cytometry; and Chuck Sherr and Fred Quelle for critical reading of the manuscript.

REFERENCES


The Alternative Reading Frame Tumor Suppressor Inhibits Growth through p21-dependent and p21-independent Pathways

Modestos Modestou, Valerie Puig-Antich, Chandrashekhar Korgaonkar, et al.

Cancer Res 2001;61:3145-3150.

Updated version

Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/61/7/3145

Cited articles

This article cites 48 articles, 24 of which you can access for free at:
http://cancerres.aacrjournals.org/content/61/7/3145.full.html#ref-list-1

Citing articles

This article has been cited by 12 HighWire-hosted articles. Access the articles at:
/content/61/7/3145.full.html#related-urls

E-mail alerts

Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions

To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions

To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.