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ARF Promotes MDM2 Degradation and Stabilizes p53: *ARF-INK4a* Locus Deletion Impairs Both the Rb and p53 Tumor Suppression Pathways

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Summary

The *INK4a-ARF* locus encodes two unrelated proteins that both function in tumor suppression. p16^{INK4a} binds to and inhibits the activity of CDK4 and CDK6, and ARF arrests the cell cycle in a p53-dependent manner. We show here that ARF binds to MDM2 and promotes the rapid degradation of MDM2. This interaction is mediated by the exon 1β–encoded N-terminal domain of ARF and a C-terminal region of MDM2. ARF-promoted MDM2 degradation is associated with MDM2 modification and concurrent p53 stabilization and accumulation. The functional consequence of ARF-regulated p53 levels via MDM2 proteolysis is evidenced by the ability of ectopically expressed ARF to restore a p53-imposed G1 cell cycle arrest that is otherwise abrogated by MDM2. Thus, deletion of the *ARF-INK4a* locus simultaneously impairs both the INK4a–cyclin D/CDK4-RB and the ARF-MDM2-p53 pathways.

Introduction

The development of human cancers is frequently associated with the inactivation of two major tumor suppression pathways represented by the retinoblastoma protein, pRB, and by p53 (Weinberg, 1995; Sherr, 1996; Levine, 1997). Via distinct mechanisms, pRB and p53 play critical roles in transducing a variety of growth inhibitory signals to the cell cycle control machinery, and their inactivation may lead to uncontrolled cell proliferation and a predisposition to neoplasia. pRB exerts most, if not all, of its growth suppressive effects in the first two-thirds of the G1 phase of the cell cycle by binding transcription factors required for initiating and progressing through S phase. When bound by pRB, these transcription factors are converted from transcriptional activators to transcriptional repressors, thereby preventing cells from exiting the G1 phase of the cell cycle (Weinberg, 1995). On the other hand, p53 is itself a transcription factor that activates the expression of genes that induce a G1 cell cycle arrest or apoptosis in response to DNA damage (Gottlieb and Oren, 1996; Ko and Prives, 1996, Levine, 1997). The concurrent inactivation of these two pathways in human cancer occurs frequently and argues that unscheduled entry into the cell cycle and escape from cell cycle arrest/apoptosis following DNA damage may be two critical events that a cell requires to become cancerous.

The regulation of p53 and pRB pathways are distinctly different. Many genotoxic stresses, particularly DNA damage, induce p53 protein levels, resulting in either cell cycle arrest or apoptosis. While inactivating mutations of p53 are common in human cancer, the molecular pathways regulating p53 abundance by individual signals have yet to be elucidated fully; however, many appear to regulate p53 posttranscriptionally, such as through governance of p53's ubiquitin-mediated proteolysis (Ko and Prives, 1996). p53's tumor suppression activity is in fact directly opposed by the human papillomavirus E6 oncoprotein through its targeting p53 for degradation (Scheffner et al., 1990, 1993). Similarly, MDM2-induced p53 degradation appears to play a critical role in regulating the level of p53 protein (Haupt et al., 1997; Kubbutat et al., 1997). *mdm2*, a proto-oncogene that is amplified in 30%–40% of sarcomas (Oliner et al., 1992), encodes a protein that can bind and inactivate the transcriptional activity of p53, resulting in abrogation of p53's antiproliferative and apoptotic effects (Momand et al., 1992; Oliner et al., 1992, 1993). Most sarcomas with *mdm2* amplification retain the wild-type *p53* gene and protein (Leach et al., 1993), suggesting that amplification of *mdm2* may be biologically equivalent to mutational inactivation of p53. Deletion of the *mdm2* gene in mice results in early embryonic lethality, which can be rescued by the simultaneous deletion of *p53*, indicating that p53 is the major target of MDM2 in development (Jones et al., 1995; Luna et al., 1995). Unlike p53, where regulation of activity appears to be primarily at the level of protein abundance, the growth-suppressive function of pRB is primarily regulated by cell cycle–dependent phosphorylations (Weinberg, 1995). Following mitosis, pRB emerges in a hypophosphorylated form, which becomes hyperphosphorylated during mid to late G1 phase and then maintains this hyperphosphorylated state throughout the remainder of the cell cycle. Hypophosphorylated pRB binds and controls a number of cellular transcription factors such as the members of the E2F family, while hyperphosphorylated pRB no longer interacts with these proteins. The kinases that have been strongly implicated as responsible for pRB phosphorylation are the cyclin-dependent kinases (CDKs), particularly the cyclin D–dependent kinases CDK4 and CDK6 (Ewen et al., 1993; Kato et al., 1993).

The activity of CDK4/6 is inhibited by association with CDK inhibitors (Sherr and Roberts, 1995). A specific inhibitor of CDK4/6, p16*INK4a*, is mutated, deleted, or down-regulated by promoter methylation in many human tumors. In human cancers, the estimated frequency of genetic alteration involving the *p16* locus is believed to be second only to alteration of *p53* (Hunter and Pines, 1994; Sherr, 1996). The extraordinarily high frequency of genetic alteration of the *INK4a* locus in human cancer may stem from its unique genomic organization. In addition to INK4a (Serrano et al., 1993), this locus encodes a second protein, ARF, whose enforced expression, like

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that of INK4a, also induces cell cycle arrest and whose disruption in mice results in development of multiple tumor types early in life (Quelle et al., 1995; Kamijo et al., 1997). The *ARF-INK4a* locus contains two unique first exons, designated 1α and 1β, which are spliced into common exons 2 and 3 (Duro et al., 1995; Mao et al., 1995; Quelle et al., 1995; Stone et al., 1995). While the exon 1α–exon 2–exon 3 transcript encodes the CDK4/6-specific inhibitor INK4a, the exon 1β–exon 2–exon 3 transcript through an **a**lternative **r**eading **f**rame encodes ARF, which bears no homology to INK4a at the amino acid level (Quelle et al., 1995). As such, homozygous deletion of the locus, which occurs frequently in a variety of human cancers, causes a loss of both the *INK4a* gene and the *ARF* gene, spawning complications in determining the individual contributions of the two genes in tumor suppression (Serrano et al., 1996; Kamijo et al., 1997). While analysis of both familial and sporadic cancer-associated mutations have indicated that *INK4a*, not *ARF*, is preferentially being targeted (Quelle et al., 1997), mice lacking p19*ARF* but retaining intact p16*INK4a* develop multiple tumors (Kamijo et al., 1997), indicating that ARF, like INK4a, is also a bona fide tumor suppressor. Unlike INK4a, which binds to and inhibits the activity of CDK4/6 (Serrano et al., 1993; Xiong et al., 1993a), ARF does not induce cell cycle arrest by acting directly as a CDK inhibitor (Quelle et al., 1995), and there is no evidence that ARF exerts its growth-inhibitory effect through the pRB pathway; instead, it requires the presence of wild-type p53 for induction of a G1 cell cycle arrest. Established cell lines that lack p53 or have p53 mutations are resistant to growth arrest by ectopic expression of ARF (Kamijo et al., 1997), suggesting that ARF may act upstream of p53. We report here that human ARF protein binds to and promotes the rapid degradation of MDM2, leading to stabilization and accumulation of p53. Our results reveal an ARF-MDM2-p53 tumor suppression pathway that parallels the INK4a–cyclin D/CDK4-pRB pathway. Deletions of the *INK4a-ARF* locus therefore effectively impair both of these two major tumor suppression pathways.

Results

Human ARF Binds to MDM2

We used the yeast two-hybrid system to search for proteins capable of physically interacting with human ARF (Fields and Song, 1989). A 0.4 kb cDNA fragment encoding the 132 amino acid residues of full-length human ARF was fused in-frame with the yeast *Gal4* DNA–binding domain (Gal4-BD). The resulting vector was cotransformed into yeast HF7c cells with a human HeLa two-hybrid cDNA library (see Experimental Procedures). Of an estimated 2.1×10^6 transformants screened, one clone was obtained that proliferated on media lacking histidine and was positive for β-galactosidase staining (Figure 1A and data not shown). Direct DNA sequencing of the rescued plasmid revealed that this clone encodes a truncated human MDM2 containing the C-terminal 284 amino acids (beginning with residue 208), suggesting that the ARF protein may interact with a C-terminal domain of MDM2. The interaction between ARF and MDM2 protein appeared to be relatively strong as indicated by

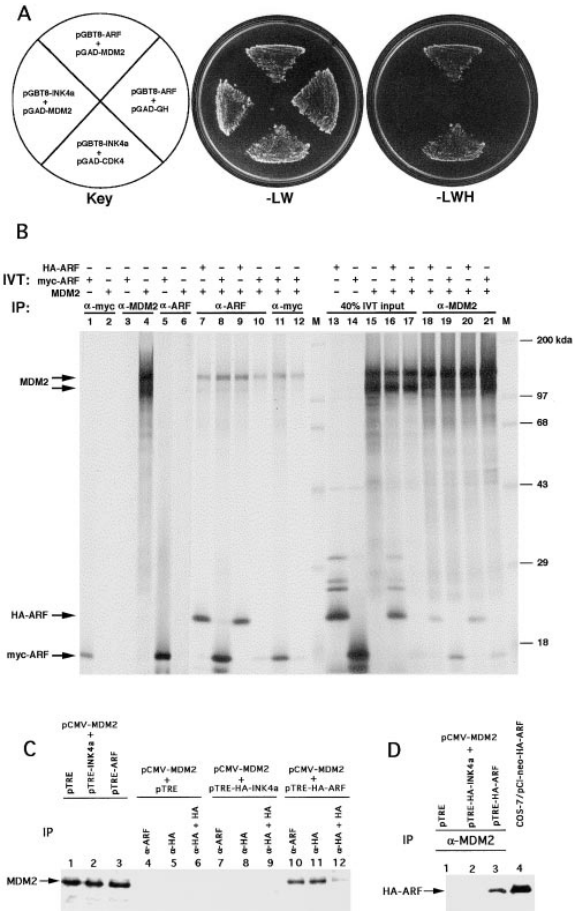

Figure 1. Human ARF Binds MDM2

(A) HF7c yeast cells were cotransformed with the indicated plasmids (key) and plated onto media lacking leucine and tryptophan (−LW) to verify the expression of both bait (Leu^+) and prey (Trp^+) plasmids; onto a −LW plate for assaying β-galactosidase activity (data not shown); or onto media lacking leucine, tryptophan, and histidine (−LWH) for examining the interaction between bait and prey proteins.

(B) Epitope-tagged (HA or myc) human ARF and MDM2 were in vitro translated in the presence of [^{35}S]methionine, either separately (lanes 1–8, 11, 13–15, 18, and 19) or together (lanes 9, 10, 12, 16, 17, 20, and 21). IVT products either directly underwent SDS-PAGE (input, lanes 13–17) or were mixed to allow complex formation and then immunoprecipitated with the indicated antibodies prior to electrophoresis.

(C) HeLa–tet off cells were cotransfected with vectors expressing human mdm2 and either HA-tagged INK4a (HA-INK4a), HA-tagged ARF (HA-ARF), or the parental pTRE vector. Two days after transfection, cell lysates were prepared and immunoprecipitated with affinity-purified antibodies to ARF (α-ARF), to HA (α-HA), or to HA antisera blocked with HA peptide (α-HA + HA). Immunoprecipitates were separated by SDS-PAGE and immunoblotted with MDM2 antisera.

(D) Mdm2 immunoprecipitates (lanes 1–3) from cells transfected as in (C) were electrophoretically separated prior to immunoblotting with an anti-ARF antibody. Lane 4 contains cell lysate from COS7 cells transfected with human HA-ARF sequences to confirm the mobility of HA-ARF in the HeLa cell lysate.

the growth of HF7c yeast cells in the presence of the histidine synthesis inhibitor 3-amino-1, 2, 3-triazole (3-AT, data not shown). ARF was unable to interact with the GAL4 DNA activation domain (negative control) expressed from the parental vector pGAD-GH. Conversely,

human INK4a fused with Gal4-BD was able to interact with CDK4 but not with MDM2 (Figure 1A).

We confirmed ARF-MDM2 binding in a cell-free system using in vitro translated proteins (Figure 1B). Two versions of the ARF protein with different N-terminal epitope tags, one containing a myc epitope (myc-ARF) and one containing an HA epitope (influenza hemagglutinin, HA-ARF), were in vitro translated using reticulocyte lysate in the presence of [^{35}S]methionine, either separately or together with full-length human MDM2. Mixtures containing [^{35}S]-labeled ARF and MDM2 proteins were immunoprecipitated with either an affinity-purified polyclonal antibody specific to ARF or with monoclonal antibodies to myc or MDM2, and the precipitates were resolved by SDS-PAGE. Human MDM2 protein, produced either by in vitro translation (Figure 1B) or ectopic expression in mammalian cells (see below), resolved by SDS-PAGE into two discrete bands. The exact nature and origin of these two forms of MDM2 protein have not been determined. No detectable differences were observed between these two forms throughout the study. Neither the myc nor the ARF antibody cross-reacted with MDM2, and likewise the MDM2 antibody did not cross-react with ARF (Figure 1B, lanes 1–6). MDM2 protein was coprecipitated with ARF by both the anti-ARF (lanes 7–10) and the anti-myc antibodies (lanes 11 and 12) whether ARF and MDM2 proteins were co-translated (lanes 9, 10, and 12) or mixed after separate translation (lanes 7, 8, and 11). Reciprocally, the ARF protein was detected in the same set of mixtures when immunoprecipitated with two different antibodies specific to MDM2; one recognizes an epitope corresponding to amino acids 154–167 (lanes 18–21), and one recognizes an epitope between residues 3–22 (data not shown).

To further confirm complex formation between ARF and MDM2 proteins, HeLa tet-off cells were transfected with plasmids directing the expression of human MDM2 (pCMV-MDM2) together with either HA epitope–tagged ARF (pTRE-HA-ARF), INK4a (pTRE-HA-INK4a), or the parental vector (pTRE). Expression of similar amounts of MDM2 protein in all the transfections was confirmed by immunoblotting of total cell lysates with anti-MDM2 antibody (Figure 1C, lanes 1–3). Protein complexes were also examined by sequential immunoprecipitation and immunoblotting (IP-Western). When total cell lysates were immunoprecipitated with an antibody specific to either ARF or HA, MDM2 protein was detected in the lysate derived from cells cotransfected with MDM2 and ARF (lanes 10 and 11) but not in cells cotransfected with MDM2 and either the parental pTRE vector (lanes 4–6) or INK4a (lanes 7–9). Coprecipitation of MDM2 with HA-ARF was efficiently blocked by the preincubation of the anti-HA antibody with a competing antigenic HA peptide (lane 12). Reciprocally, the ARF, but not the INK4a, protein was detected in anti-MDM2 immunoprecipitates derived from cells cotransfected with MDM2 (Figure 1D).

ARF Interacts with MDM2 via an Exon 1β–Encoded Domain

The *ARF* and *INK4a* genes share common exons 2 and 3, but each contains a unique exon 1. Deletion analysis has mapped the cell cycle arrest activity of ARF within

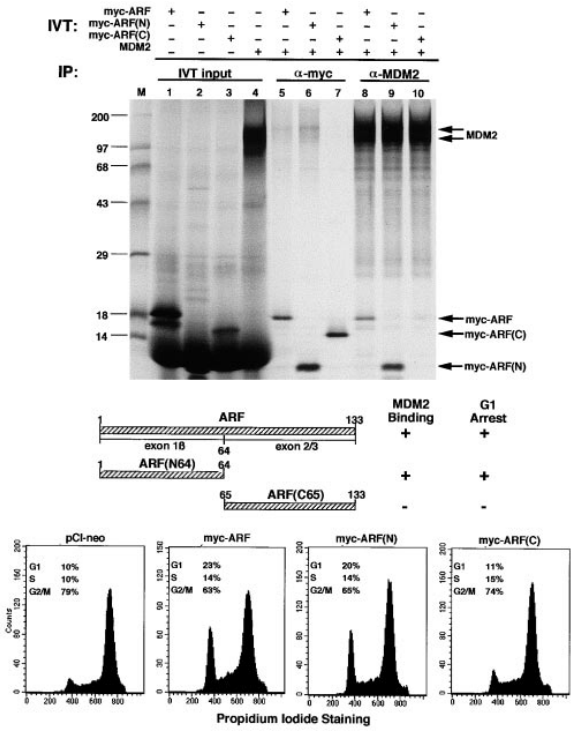

Figure 2. ARF Binds to MDM2 through an Exon 1β–Encoded N-Terminal Domain

Myc epitope–tagged full-length ARF (myc-ARF), the exon 1β–encoded N-terminal domain (myc-ARF[N]), and the exon 2-3–encoded C-terminal domain [myc-ARF(C)] of human ARF were in vitro translated and labeled with [^{35}S] and then mixed with similarly produced full-length human MDM2. Mixtures were immunoprecipitated with indicated antibodies followed by SDS-PAGE and autoradiography. U2OS cells were transfected with the indicated plasmids containing the mutant ARF proteins described above in addition to CD20. Forty-eight hours later transfected cells were analyzed for cell cycle position after staining with propidium iodide following 10 hr of nocodazole treatment. Schematic representation of the deletion constructs and their ability to bind to MDM2 and cause a G1 arrest are provided.

exon 1β, which encodes 64 amino acids residues. The exon 1β domain by itself can induce a G1 cell cycle arrest with a potency similar to if not higher than that of full-length ARF, and deletion of exon 1β resulted in a mutant ARF that was unable to arrest the cell cycle (Quelle et al., 1997). To identify the domains within ARF that are required for binding to MDM2, we constructed two ARF mutants, one containing the N-terminal 64 amino acids encoded by exon 1β and the other lacking the exon 1β encoded N-terminal 64 amino acids, and tested their ability to bind MDM2. Because our anti-ARF C-terminal peptide antibody does not recognize the N-terminal domain, we added a myc epitope tag to the amino terminus of both mutants (myc-ARF[N] and myc-ARF[C], respectively). In vitro translated, [^{35}S]-labeled wild-type or mutant ARF protein was mixed with similarly produced full-length MDM2 protein and reciprocally immunoprecipitated with antibodies to either MDM2 or the myc epitope (Figure 2, top). We first confirmed the expected effects on the cell cycle by the ectopic expression of each ARF construct. As previously observed, expression of the exon 1β–encoded domain

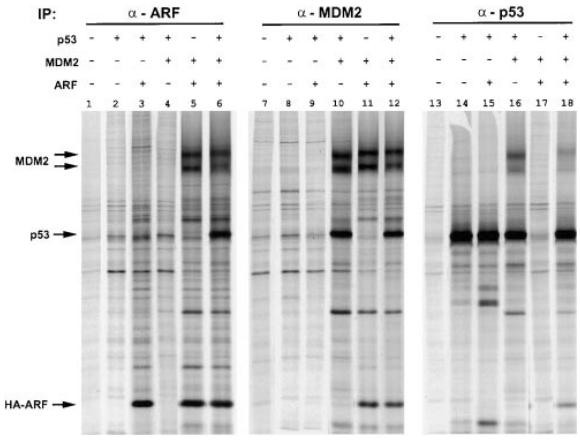

Figure 3. Human ARF, MDM2, and p53 Form a Ternary Complex

HeLa cells were transiently transfected with combinations of plasmids directing expression of human ARF, MDM2, and/or p53. Two days after transfection, the cells were metabolically labeled with [^{35}S]methionine, and the lysates were immunoprecipitated with antibodies to ARF, MDM2, and p53. Immunoprecipitated proteins were separated by SDS-PAGE and visualized by autoradiography.

is sufficient to cause a G1 cell cycle arrest with similar potency to that caused by the full-length ARF (Figure 2, bottom). While the C-terminal domain mutant did not exhibit any detectable interaction with MDM2 (lanes 7 and 10), the N-terminal domain of ARF binds to MDM2 with similar affinity as full-length ARF (compare lanes 5 and 6, 8 and 9), indicating that the N-terminal 64 amino acids encoded by exon 1β are both required and sufficient for binding to MDM2.

ARF Forms a Ternary Complex with MDM2 and p53

MDM2 binds p53 through an N-terminal domain (Chen et al., 1993; Oliner et al., 1993). The observation that ARF binds to a C-terminal domain of MDM2 prompted us to test whether ARF can form a ternary complex with MDM2 and p53. HeLa cells were transfected with combinations of plasmid vectors expressing ARF, MDM2, and/or p53. Transfected cells were metabolically labeled with [^{35}S]methionine, and the cell lysates were immunoprecipitated with antibodies specific to each of the individual proteins. All three antibodies specifically recognized their target proteins (Figure 3, lanes 3, 10, and 14) and did not cross-react with the other two proteins (lanes 4, 9, and 17). Both binary ARF–MDM2 (lanes 5 and 11) and MDM2–p53 (lanes 10 and 16) complexes can be readily precipitated using antibodies to either relevant protein. In cells triply transfected with plasmids expressing all three proteins, an ARF–MDM2–p53 ternary complex was formed as determined by the presence of ARF in anti-p53 immunoprecipitates (lane 18) and reciprocally p53 in anti-ARF precipitates (lane 6). In the absence of MDM2, ARF and p53 association was not detectable (lanes 3 and 15), indicating that the ARF-p53 association is bridged by MDM2.

ARF Promotes the Rapid Degradation of MDM2

The main function of MDM2 is to regulate p53 by complexing with and promoting the rapid degradation of p53. Formation of a ternary ARF–MDM2–p53 complex suggests that if ARF regulates MDM2 function, it may not involve simple competitive disruption of MDM2's association with p53. Therefore, we examined the possibility that ARF affects the stability of MDM2. HeLa cells were transfected with plasmid vectors expressing MDM2 with or without ARF or mdm2 and p53 with and without ARF, and the half-life of MDM2 was determined by pulse-chase labeling (Figure 4A). Ectopically expressed MDM2 alone has a half-life of approximately 90 min in HeLa cells. Coexpression of MDM2 with ARF dramatically reduced the half-life of MDM2 protein to approximately 30 min regardless of the presence or absence of p53 (Figure 4B). To determine whether the ARF-dependent shortened half-life of MDM2 decreased the steady-state level of MDM2 protein, total cell lysate derived from transfected HeLa cells was separated by SDS-PAGE, transferred to a nitrocellulose filter, and appropriate sections were immunoblotted with antibodies specific to each transfected protein. Cotransfection of MDM2 with ARF (Figure 4C, lane 4), but not with INK4a (lane 5), significantly reduced the steady-state level of MDM2 protein. The apparent constant steady-state level of p53 protein resulting from the transfections in this experiment was by design. By transfecting with high levels of p53 expressing vector, we could determine whether the change in MDM2 protein abundance mediated by ARF is p53 protein level independent.

Of potential significance is a distinct mobility shift of the MDM2 protein upon coexpression with ARF protein (Figure 3, compare lanes 10 with 11 and 12; Figure 4A, compare lanes 1–6 with lanes 7–12; lanes 13–18 with lanes 19–24). We have not determined the nature of this ARF-promoted MDM2 modification and whether this modification regulates the stability of MDM2.

Degradation of MDM2 by ARF Leads to p53 Stabilization and Accumulation

MDM2 promotes the rapid degradation of p53 (Haupt et al. 1997, Kubbutat et al., 1997). To test whether ARF-promoted MDM2 degradation leads to p53 stabilization and accumulation, we determined the half-life of p53 in cells cotransfected with either MDM2 or MDM2 plus ARF (Figure 5A). The half-life of p53 ectopically expressed in HeLa cells is approximately 3 hr, shorter than the estimated 7 hr half-life determined in Saos-2 cells (Kubbutat et al., 1997). We presume this change in p53's half-life is due to an E6-activated p53-degradation activity present in HeLa cells. Ectopic expression of p53 apparently overides the E6-mediated p53 degradation activity, as evidenced by the accumulation of p53 (see below). This is consistent with previous observations that treatment of HPV-positive cells with genotoxic agents, such as UV and mitomycin, resulted in an increase of p53 protein and its transactivating activity (e.g., Butz et al., 1995). Cotransfection of a p53 expressing plasmid with a plasmid expressing MDM2 reduced the half-life of p53 from 3 hr to about 60 min. Triple transfection of plasmids expressing p53, MDM2, and ARF completely restored the half-life of p53 to 3 hr (Figure 5B). To test whether inhibition of MDM2-promoted p53 destabilization by ARF results in p53 accumulation, the steady-state level

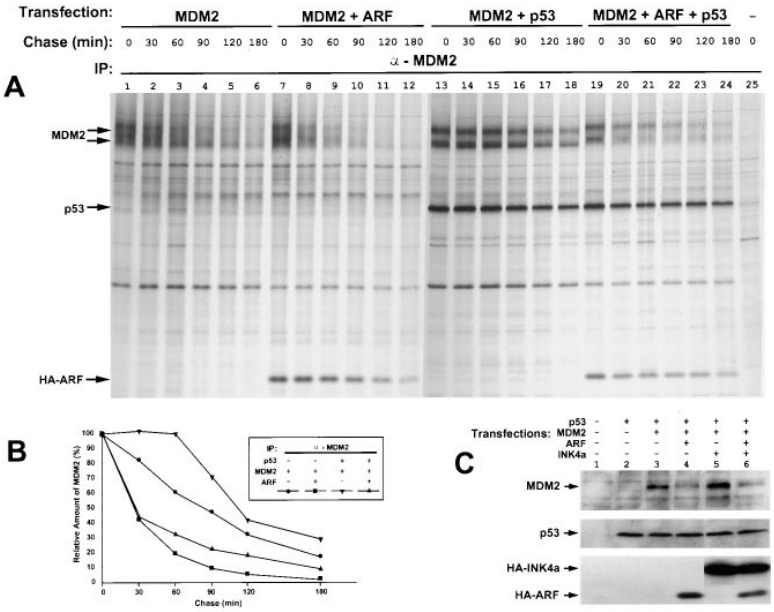

Figure 4. ARF Promotes Rapid Degradation of MDM2

(A) HeLa cells were transfected with the indicated plasmids. Twenty-four hours after transfection, cells were pulsed with [^{35}S]methionine for 2 hr, then chased with fresh "cold" media for the indicated length of time, and the lysates were immunoprecipitated with an antibody specific to MDM2. MDM2 precipitates were separated by SDS-PAGE and visualized by autoradiography.

(B) The amount of labeled MDM2 protein at each time point was quantitated on a PhosphoImager (Molecular Dynamics, ImageQuant software version 3.3), normalized relative to the amount of radiolabeled MDM2 present in cells following the 0 min chase, and plotted versus chase time for HeLa cells transfected with the indicated plasmids.

(C) Lysates of HeLa cells transiently transfected with combinations of plasmids expressing p53, MDM2, HA-tagged human ARF, and INK4a were electrophoretically separated and immunoblotted with antibodies recognizing mdm2, p53, or the HA epitope.

of p53 was determined by IP-Western (Figure 5C). HeLa cells express nearly undetectable levels of endogenous p53 (lane 4). Cotransfection of MDM2 with p53 reduced p53 to nearly background levels (compare lanes 2 and 4). Transfection of ARF together with p53 and MDM2 restored p53 steady-state levels to a level similar to that seen in singly transfected cells (compare lanes 1 and 3), indicating that ARF blocks MDM2-promoted p53 degradation. We purposely used 5:5:1 ratio of ARF, MDM2, and p53 expressing plasmids in this experiment to ensure that adequate levels of MDM2 and ARF would be present to affect p53 levels.

We also examined the stability of ARF by pulse-chase labeling of HeLa cells transfected with ARF sequences. Transfected ARF has a half-life of approximately 90 min in HeLa cells similar to that of MDM2 (data not shown), suggesting the possibility that ARF like MDM2 may be regulated by proteolysis. It is interesting to note that unlike ARF, INK4a is a protein with a long half-life (with little change over a 3 hr half-life experiment) (Parry et al., 1995), further underscoring the distinction between these two proteins. The half-life of ARF protein did not change significantly in the presence of either ectopically expressed MDM2 or MDM2 plus p53 (data not shown).

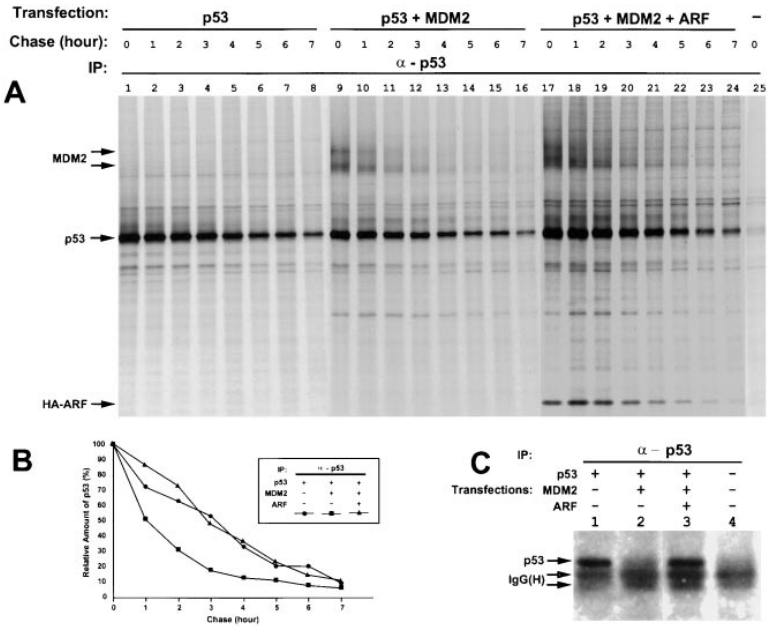

Figure 5. ARF Abrogates MDM2-Promoted p53 Degradation

(A) HeLa cells were transiently transfected with *p53, p53* and *mdm2*; or *p53*, *mdm2*, and *ARF* expression plasmids as indicated. Twenty-four hours after transfection, cells were pulsed with [^{35}S]methionine for 2 hr and then chased for the indicated length of time. Cell lysates were immunoprecipitated with an antibody specific to p53. p53 immunoprecipitates were separated electrophoretically by SDS-PAGE and visualized by autoradiography.

(B) The amount of labeled p53 protein at each time point was quantitated on a PhosphoImager (Molecular Dynamics, ImageQuant software version 3.3), normalized relative to the amount of radiolabeled p53 present in cells following the 0 min chase, and plotted versus chase time for HeLa cells transfected with the indicated plasmids.

(C) HeLa cells were transiently transfected with plasmids expressing human p53 alone (lane 1), p53 and MDM2 (lane 2), or p53, MDM2, and ARF (lane 3). Lane 4 represents untransfected HeLa cells. Each individual transfected cell lysate was immunoprecipitated with p53 antisera prior to electrophoretic separation, immobilization of the proteins, and immunoblotting with p53 antisera.

ARF Reverses MDM2's Abrogation of p53-Mediated Growth Arrest

Forced overexpression of MDM2 is able to completely abolish the G1 cell cycle arrest caused by p53 (Chen et al., 1994; 1996). Given the effects of ARF expression on the stability of MDM2 and p53 proteins, we sought to determine if ARF could reverse MDM2's abrogation of p53-induced cell cycle arrest. The human U2OS cell line, a human osteosarcoma line that contains wild-type p53, was transfected with combinations of plasmids driving expression of p53, MDM2, and ARF. Cells were simultaneously transfected with the cell surface marker CD20 to allow selection of transfected cells. Following transfection, a portion of the cells was analyzed for cell cycle distribution by flow cytometric analysis of CD20-positive cells, while another portion was analyzed for the expression of individual proteins. Consistent with previous reports (Chen et al., 1996), ectopically expressed p53 caused cell cycle arrest in G1, and this p53-mediated G1 arrest was overcome by MDM2 (Figure 6A, 6B). When ARF was cotransfected into cells overexpressing p53 and MDM2, it restored the G1 arrest to the level seen in cells singularly transfected by p53. Analysis of protein expression by immunoblotting revealed that p53 transfection resulted in an increase in the steady-state level of endogenous p21 (Figure 6C). The increase in p21 is most likely due to transcriptional activation by p53, since cotransfection of MDM2 resulted in a decrease in p21 protein levels to that seen in cells transfected with vector alone. Ectopic expression of ARF in addition to p53 and MDM2 restored the p53-induced p21 protein levels and was associated with a decrease in the steady-state levels of the MDM2 protein similar to that observed in HeLa cells (Figure 4C). We noticed that the steady-state level of p53 was not significantly changed in cells transfected with MDM2 or in cells transfected with MDM2 and ARF in addition to p53 (Figure 6C, lanes 2–4). This data is consistent with the notion that MDM2 can affect p53's transactivating activity without altering p53 protein levels, through repression of p53 mediated transcription by either masking the activity domain of p53 or by directly interfering with the basal transcriptional machinery (Momand et al., 1992, Thut et al., 1997)

Although we only determined the steady-state level of each protein, not their half-lives, this result is consistent with our finding in HeLa cells that ARF can decrease the steady-state levels of MDM2 by promoting its degradation. The simplest explanation for the cancellation of MDM2-imposed abrogation of p53-mediated arrest by ARF is that the rapid degradation of MDM2 by ARF leads to p53 stabilization and accumulation.

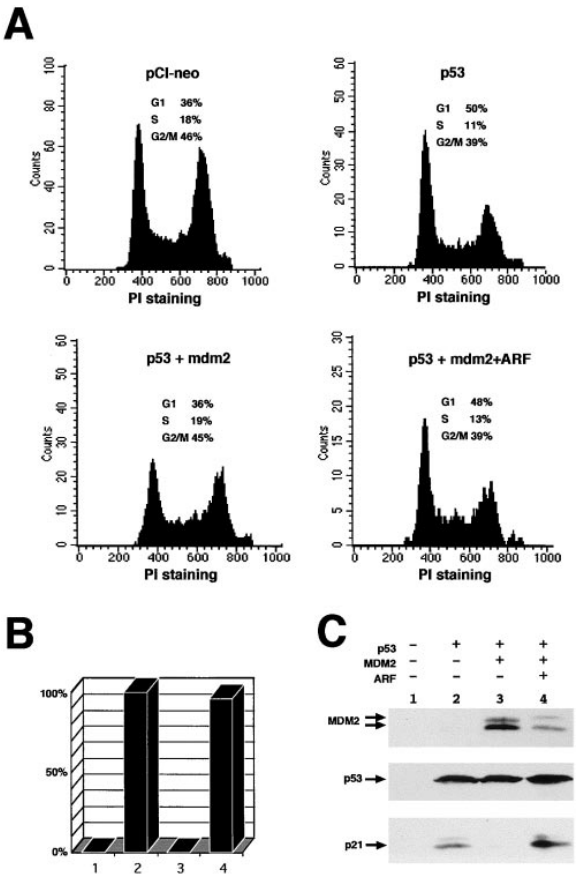

Figure 6. Human ARF Restores MDM2-Abrogated, p53-Mediated Cell Cycle Arrest

(A) U2OS cells were transiently transfected with the CD20 cell surface marker and pCI-neo; p53; p53 and mdm2; or p53, mdm2, and human ARF. The fraction of transfected cells (as determined by expression of CD20) in the G1 phase of the cell cycle was compared between the transfections.

(B) The G1 cell cycle arrest caused by p53 was standardized to 100% (the actual increase in the G1 fraction caused by p53 was 14%). Cotransfection of mdm2 with p53 completely reversed the G1 arrest seen with the transfection of p53 alone. Expression of human ARF in addition to p53 and mdm2 restored the G1 cell cycle arrest seen with p53 alone. A minimum of 4,000 CD20-positive cells were analyzed for each transfection.

(C) Lysates from cells transfected with the indicated plasmids, in addition to CD20, were electrophoretically separated before immunoblotting with α-MDM2, α-p53, or α-p21.

Discussion

The *ARF-INK4a* locus encodes two unrelated proteins both capable of inducing cell cycle arrest when overexpressed (Serrano et al., 1993; Quelle et al., 1995). The INK4a protein inhibits cell cycle progression by binding to and inhibiting the activity of cyclin D–dependent kinases 4 and 6 (CDK4 and CDK6), thereby maintaining the pRB protein in its growth suppressive, hypophosphorylated state. The ARF protein, when ectopically expressed, causes cell cycle arrest in cells containing wild-type p53 but not in p53-deficient cells (Kamijo et al., 1997), suggesting that ARF may act upstream of p53. Here, we present evidence that the ARF protein can stabilize the p53 protein by binding to and promoting the degradation of MDM2, providing a molecular mechanism for ARF function in cell cycle control and tumor suppression.

Destabilization of MDM2 by ARF precluded detection of in vivo complex formation between these two proteins. However, four different assays all indicate that this interaction is direct and not bridged by another molecule: the yeast two-hybrid assay, reconstitution in insect cells through baculovirus expression, an in vitro binding assay, and in vivo transfection of mammalian cells (see also Pomerantz et al., 1998 [this issue of *Cell*]).

The binding domains for the ARF–MDM2 interaction have been mapped to the N-terminal and C-terminal regions of ARF and MDM2, respectively. Ectopic expression of the exon 1β–encoded N-terminal region of murine ARF has been previously shown to be both necessary and sufficient to cause cell cycle arrest (Quelle et al., 1997). Our finding that ARF binds MDM2 through exon 1β–encoded sequences domain is entirely consistent with and further supports the biological activity of this domain in arresting the cell cycle. MDM2 binds to p53 via its N-terminal domain, and deletion of the C-terminal portion has little effect on the binding of MDM2 to p53 (Chen et al., 1993; Oliner et al., 1993), indicating that the highly conserved central and C-terminal regions may encode a separable function(s). The C-terminal 284 residues of MDM2 is sufficient to interact with ARF (Figure 1A), assigning a possible function to this ARF-binding region as important in regulation of MDM2 stability and abundance.

The functional significance of the ARF–MDM2 interaction is not fully understood at present. One consequence of the ARF–MDM2 interaction is promotion of MDM2 protein degradation. The half-life of ectopically expressed MDM2 protein in HeLa cells of approximately 90 min was reduced to 30 min by cotransfection of an equal amount of plasmid vector expressing ARF (Figure 4A). Reduction of the half-life of MDM2 by ARF is associated with a decrease in the steady-state level of MDM2 protein to nearly background level in transfected cells, indicating the potency of ARF in promoting the degradation of MDM2. Consequently, the p53 protein, which is destabilized by overexpression of MDM2, is stabilized by the coexpression of ARF, restoring the p53 level to that seen in singly transfected cells (Figure 5C). We further note that ARF itself is a short-lived protein with its estimated half-life being approximately 90 min in transfected HeLa cells, suggesting the possibility that the abundance of ARF may also be subjected to regulation at the level of protein stability.

The biochemical mechanism underlying ARF-promoted MDM2 degradation, as in the case of MDM2-promoted p53 degradation, remains to be elucidated. Whether the degradation of MDM2, like that of p53 (Maki et al. 1996), is also mediated by an ubiquitin-dependent pathway is not known. ARF-promoted MDM2 degradation could be indirect (e.g., by regulating the modification of MDM2 and p53) or direct (e.g., by acting as an E3-like ubiquitin ligase to target MDM2, analogous to HPV E6–promoted p53 degradation). A possible complication stems from the presence in HeLa cells of HPV-16 E6 protein, which can utilize the cellular proteolytic system to target p53 for ubiquitin-dependent degradation (Scheffner et al., 1990, 1993). Although there is no evidence that E6 could have a similar effect on the stability of MDM2, we cannot firmly exclude the possibility that E6 may have contributed to ARF-promoted MDM2 degradation through an as yet unknown mechanism. Several observations, however, argue against such a possibility. First, binding of ARF to MDM2 does not require any other protein, and expression of E6 in HeLa cells does not disrupt this interaction. Second, E6 targets p53 for degradation by promoting the interaction of p53 with E6AP (a protein containing ubiquitin ligase activity). In the absence of E6, E6AP does not bind to p53 (Huibregtse et al., 1991; Scheffner et al., 1993). On the other hand, MDM2 promotion of p53 degradation requires direct binding of MDM2 to p53 and is independent of both E6 and E6AP (Haupt et al., 1997; Kubbutat et al., 1997). This suggests that E6 and MDM2 utilize different mechanisms to selectively target p53 for degradation and that ARF stabilizes p53 independently of E6. Third, if the increase in MDM2 degradation by ARF required or was enhanced by E6, it would suggest that E6 itself is associated with an activity that promotes MDM2 degradation. In essence, that would lead to an E6-promoted p53 stabilization, rather than degradation as has been observed. Finally, transfection of U2OS cells, a human osteosarcoma cell line that does not contain E6, by p53 resulted in a cell cycle arrest that was abrogated by cotransfection of p53 and MDM2. The p53-mediated arrest was restored by triple transfection with p53, MDM2, and ARF (Figure 6). In these transfected cells, ectopic expression of p53 resulted in an increase in p21, cotransfection of p53 with MDM2 reduced p21 to background level, and triple transfection with ARF restores p21 back to the level seen in p53–singly transfected cells. Although we only determined the steady-state level of each protein, not their half-lives, this result supports and is consistent with the finding made in HeLa cells that ARF promotes MDM2 degradation. Our results, however, do not exclude the possibility that ARF may regulate MDM2 through other mechanisms in addition to promoting its degradation. Intriguingly, ARF, MDM2, and p53 can form a stable ternary complex, likely bridged by MDM2 via its two separate domains, which can independently interact with p53 and ARF (Figure 3). It is conceivable that ARF may be able to ablate MDM2's functional repression of p53-mediated transcription (Thut et al., 1997).

Normally, p53 protein is kept at a low concentration in a cell by its relatively short half-life. Several types of cellular stresses, particularly DNA damage, result in a rapid increase in p53 concentration. The mechanism by which these genotoxic stresses induces p53 accumulation is not well understood but likely involves lengthening p53's half-life. The major function of MDM2 in vivo appears to be regulation of p53, as evidenced by the rescue of embryonic lethality of mdm2-deficient mice by codeletion of p53 (Jones et al., 1995; Luna et al., 1995). The p53 protein is significantly destabilized by the overexpression of MDM2 (Haupt et al., 1997; Kubbutat et al., 1997) (Figure 5A), leading to the postulation that MDM2 may play a role in maintaining low levels of p53 in normal cells as well as in removing p53 during recovery from stress-induced arrest through a feedback regulatory loop. While it is tempting to speculate that ARF may be activated by stress to cause MDM2 degradation and p53 accumulation, it has been observed that induction of p53 and p21 by γ irradiation does not depend on ARF (Kamijo et al., 1997). This indicates that, at least in the case of DNA double-strand breaks produced by γ irradiation, ARF plays at most a redundant, if not a minor, role in mediating p53 accumulation. Therefore, as with INK4a, elucidating the cellular signals that regulate the level or activity of ARF remains a major challenge.

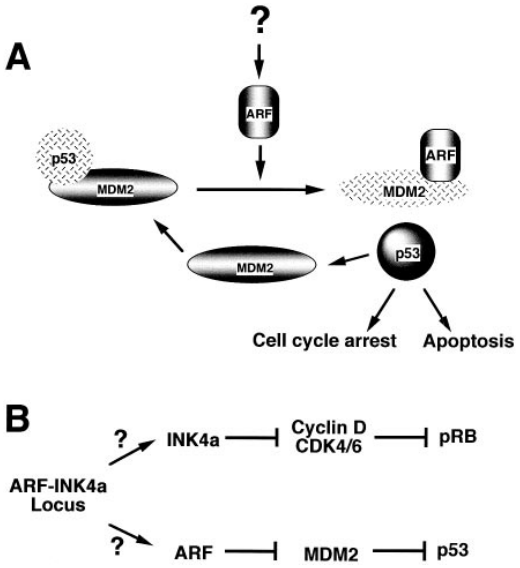

Figure 7. INK4a and ARF Pathways

(A) ARF, via an exon 1β-encoded N-terminal domain, binds to a C-terminal portion of MDM2 and promotes its degradation (indicated by stippled appearance). MDM2 via an N-terminal domain forms a complex with and promotes the degradation of p53. ARF-promoted MDM2 degradation is also associated with ARF-dependent MDM2 modification, but the significance of this modification in regulating the degradation of MDM2 is not clear. p53 is also a phosphoprotein, and phosphorylation of p53 by DNA-dependent protein kinase (DNA-PK) following DNA damage hinders its interaction with MDM2 (Shieh et al., 1997). The *mdm2* gene itself is activated by p53, creating an autoregulatory feedback loop (Barak et al., 1993, 1994; Wu et al., 1993).

(B) Comparison of the ARF-MDM2-p53 pathway and the INK4a–cyclin D/CDK4,6-pRB pathway. The regulation of both ARF and INK4a is not known.

Our finding that ARF stabilizes p53 by promoting the degradation of MDM2 reveals a tumor suppression pathway that involves two tumor suppressors, *ARF* and *p53*, both of which are frequently inactivated in many types of human cancers, and a proto-oncogene, *mdm2*, that is genomically amplified with high frequency in human sarcomas (Figure 7A). There is a striking similarity between this pathway and another major tumor suppression pathway (Figure 7B) INK4a–cyclin D1/CDK4-pRB that similarly includes two frequently mutated tumor suppressors, INK4a and pRB, and a proto-oncogene, *cyclin D1*, which is amplified in several types of human cancers (Lammie and Peters, 1991; Hunter and Pines, 1994; Sherr, 1996). Amusingly, both pathways involve two steps of negative regulation that transduce signals as stop-go-stop for the cell cycle. Cancer-associated point mutations in the *ARF-INK4a* locus have so far been found to preferentially, if not exclusively, impinge on *INK4a* (Quelle et al., 1997). No point mutations within ARF's exon 1β have been detected in primary tumors or tumor-derived cell lines. Therefore, point mutations and biallelic deletions are functionally distinct and may have been selectively targeted during tumor development: while the INK4a mutations selectively inactivate the Rb pathway, the deletion of the *ARF-INK4a* locus impairs both Rb and p53 pathways.

Experimental Procedures

Plasmid Constructs

Exon 1β of human ARF was amplified from human placental cDNA by polymerase chain reaction (PCR) using the following oligonucleotide primers based on published human ARF sequence (Duro et al., 1995; Mao et al., 1995; Stone et al., 1995): E1S (sense) 5′-GGAATTCCCATGGTGCGCAGGTTCTTGGTGACC-3′ and E1A (antisense) 5′-CATCATCATGACCTGGTCTTCTAGGAAGCGGCTGCT-3′. EcoRI and NcoI restriction sites were incorporated into the sense primer (underlined) to facilitate subsequent cloning. Exons 2 and 3 were PCR amplified from human *INK4a* cDNA using the following primers (Serrano et al., 1993): E2S (sense) 5′-CTAGAAGACCAGGTCATGATGATGGGCAGC-3′ and E2A (antisense) 5′-CGATGAATTAAGCTTGAGCTCGG-3′ (a natural HindIII site present in the 3′ UTR of ARF and INK4a is underlined). PCR conditions were: 1 μM each oligonucleotide, 2 U Taq polymerase, and 1X PCR buffer (Perkin-Elmer, Roche). For amplification of exon 2–3, dimethylsulfoxide (DMSO) was added to a final concentration of 5%. Thirty cycles of amplification were completed using the following cycling temperatures: denaturation for 30 s at 95°C, annealing for 60 s at 55°C for exon 1β or 50°C for exon 2–3, and elongation for 90 s at 72°C. The amplified exon 1β and exon 2-3 were mixed in a 1:1 ratio and reamplified using sense E1S and antisense E2A primers under the same conditions as for exon 2 PCR except that the annealing temperature was 45°C. The PCR product containing full-length human ARF coding sequence was first subcloned into a GST fusion expressing vector, pGEX-KG, after restriction with NcoI and HindIII yielding pGEX-ARF, which was verified by DNA sequencing. From pGEX-ARF, a series of ARF derivative plasmids were constructed by subcloning into pCI-neo (Promega) and adding an amino terminal HA epitope–tag into the NcoI EcoRI site of pCI-neo or by subcloning into pcDNA3-myc (a modified pcDNA3 vector that contains a myc epitope tag downstream of a CMV and a T7 promoter, InVitrogen), to yield pCI-neo-HA-ARF and pcDNA3-myc-ARF that were used for both in vitro translation using T7 RNA polymerase and expression in mammalian cells from the CMV promoter. The HA-ARF fragment was further subcloned from pCI-neo-HA-ARF into the pTRE vector (Clontech, Palo Alto, CA) that contains a CMV promoter under the control of a tetracyclin response element to yield pTRE-HA-ARF, which is suitable for expression in tetracyclin-inducible HeLa cells (HeLa-tet off, Clontech). ARF deletion mutants were constructed by PCR using pcDNA3-myc-ARF as a template. The two pairs of primers used were as follows: 5′-TAATACGACTCACTATAGGG-3′ (sense, a T7 primer) and 5′-TTACTCGAGTCATGGTCTTCTAGGAAGCGGCTGCTG-3′ (antisense, a XhoI site incorporated for subsequent subcloning, is underlined) to generate a fragment corresponding to the N-terminal 64 amino acids encoded by exon 1β; 5′-ATAGAATTCGGTCATGATGATGGGCAGCGC-3′ (sense, an EcoRI site is underlined) and E2A (antisense) to generate a fragment corresponding to the C-terminal 68 residues lacking exon 1β. Both PCR products were cloned into pcDNA3-myc to yield pcDNA3-myc-ARF(N) and pcDNA3-myc-ARF(C), respectively. All ARF plasmids were confirmed by direct sequencing of isolated plasmids.

Yeast Two-Hybrid Assay

The cDNA sequence encoding full-length human ARF was cloned into pGBT8, a modified version of pGBT9, in frame with the DNA-binding domain of *Gal4*. This construct was cotransformed into HF7c yeast with a HeLa cDNA library cloned into pGADGH (Clontech) as previously described (Guan et al., 1994). An estimated 2.1×10^6 transformants were screened. Yeast containing interacting proteins was identified by growth on media lacking leucine, tryptophan, and histidine and confirmed by assaying for β-galactosidase activity. Plasmids were rescued from positive yeast colonies and confirmed by cotransforming into HF7c cells with pGBT8-ARF and testing their growth on the selection media (−LWH) with and without 5 mM 3-aminotriazole (3-AT). The cDNA insert of rescued plasmids was determined by DNA sequencing.

Cell Culture, Transfection, and FACS Analysis

All cells, unless otherwise specified, were obtained from the ATCC. HeLa Tet-Off cells were obtained from Clontech. All cells were cultured in a 37°C incubator with 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum

(FBS). Cell transfections were carried out using the LipofectAMINE reagent according to the manufacturer's instructions (GIBCO-BRL). Cells were plated at a low density (approximately 3×10^5 cell/100 mm dish or 1×10^5 cells/60 mm dish) and allowed to grow to 30%–40% confluence (24–36 hr after seeding) at which time they were transfected using lipofectamine in a total volume of 6 ml Optimem-1 media (GIBCO-BRL). The cells were transfected with various amounts of the individual plasmids DNA as indicated in the figure legend for each experiment, and the total amount of DNA transfected was adjusted to 10 μg with pcDNA3 (Invitrogen) or pCI-neo (Promega) for each 100 mm dish or 3 μg for each 60 mm dish. Following a 5 hr incubation with the DNA/lipid mixture, the cells were washed with PBS before replenishing with growth media. The cells were harvested 24 hr posttransfection and lysed in a NP-40 buffer (Jenkins and Xiong, 1995). For cell cycle arrest analysis, cells were cotransfected with the indicated vectors (10 μg) and CMV-CD20 (1 μg) as described (Yarbrough et al., 1996). Forty hours posttransfection, cells were harvested by trypsinization, washed in PBS, stained with FITC-conjugated anti-CD20 antibody (Becton-Dickinson) for 30 min at 4°C, and then fixed in 70% ethanol as described (Yarbrough et al., 1996). After washing cells once with PBS containing 1% BSA, the DNA was stained with propidium iodide (50 μg/ml) containing 250 μg/ml of ribonuclease A. Flow cytometry analysis was conducted using a Becton-Dickinson FACScan. CD20 was used as a marker for analysis of transfected cells. DNA content from at least 4,000 CD20-positive cells is presented in the DNA histograms. Recombinant baculovirus expressing HA-tagged human ARF was constructed using the BAC-to-BAC baculovirus expression system (GIBCO-BRL). Baculoviruses expressing human MDM2 and p53 were kindly provided by Dr. J. Chen (Louisiana State University, New Orleans) and Dr. B. Merrick (National Institute of Environmental Health Science, Research Triangle Park).

Immunochemistry Procedures and Antibodies

Procedures for [^{35}S]methionine metabolic labeling, immunoprecipitation, and immunoblotting have been described previously (Jenkins and Xiong, 1995). Procedures for production, infection, and labeling insect Sf9 cells have also been described previously (Xiong et al., 1993b). Rabbit polyclonal anti-human ARF antibody was produced in male New Zealand rabbits against the synthetic peptide CGRARC LGPSARGPG with the underlined region corresponding to amino acid residues 119–132 at the C terminus of human ARF (Mao et al., 1995; Stone et al., 1995). The cysteine residue was added to the N terminus of the peptide to facilitate covalently coupling the peptide to activated keyhole limpet hemocyanin (Pierce, Rockford, IL), which was then used to immunize rabbits. Antibodies were affinity purified using the antigen peptide and a Sulfolink column following the manufacturer's instructions (Sulfolink Kit, Pierce). A detailed characterization of this antibody will be described elsewhere (C. G. Shores and W. G. Y., unpublished data). Affinity-purified monoclonal antibody to p53 (clone PAb421, Oncogene Science, Uniondale, NY), to myc (clone 9E10.3, NeoMarker, Fremont, CA), to HA (clone 12CA5, Boehringer Mannheim, Indianapolis, IN), to human MDM2 (SMP14, Santa Cruz, CA), and an affinity-purified polyclonal antibody raised against a amino acids 3-22 of human MDM2 (N-20, Santa Cruz) were purchased commercially.

Pulse-Chase Experiment

HeLa cells (1×10^5) were seeded onto a 60 mm plate and were transfected after overnight culture with a total of 3 μg of appropriate plasmid DNA. Twenty-four hours posttransfection, cells were pulse-labeled with [^{35}S]methionine for 2 hr, washed twice with prewarmed 1× PBS, and chased by culturing in DMEM/10% FBS media for the time indicated in each figure. Lysates from pulse-chase–labeled cells were immunoprecipitated as indicated.

In Vitro Transcription, Translation, and Binding Assays

Coupled in vitro transcription and translation reactions were performed using the TNT kit following the manufacturer's instructions (Promega). For in vitro binding assays, translated proteins were mixed together and further incubated at 30°C for 30 min in the same reticulocyte lysate. At the end of the incubation, 200 μl NP-40 lysis buffer (Jenkins and Xiong, 1995) was added to each binding reaction followed by immunoprecipitation with appropriate antibodies.

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