CARF Is a Novel Protein That Cooperates with Mouse p19\textsuperscript{ARF} (Human p14\textsuperscript{ARF}) in Activating p53*

The INK4a locus on chromosome 9p21 encodes two structurally distinct tumor suppressor proteins, p16\textsuperscript{INK4a} and the alternative reading frame protein, ARF (p14\textsuperscript{ARF} in mouse and p14\textsuperscript{ARF} in human). Each of these proteins has a role in senescence of primary cells and activates pathways for cell cycle control and tumor suppression. The current prevailing model proposes that p19\textsuperscript{ARF} activates p53 function by antagonizing its degradation by MDM2. It was, however, recently shown that stabilization of p53 that requires nuclear-cytoplasmic shuttling of p53-MDM2 in the nucleolus and thereby preventing the degradation of p53 (10–16). ARF expression is regulated by p53-dependent kinases (9). ARF negatively regulates MDM2-mediated inactivation of the retinoblastoma protein (pRB) by cyclin-dependent kinases (8). ARF represses E2F function and ARF expression. ARF therefore provides a mechanism whereby inactivation of Rb and release of E2F lead to the stabilization and functional activation of p53, linking the Rb and p53 pathways (6, 17). Therefore, functional regulation of ARF is critical for cell cycle control in response to a variety of cellular and environmental signals. The current prevailing model proposes that ARF functions by sequestering MDM2 in the nucleolus and thereby preventing the degradation of p53 that requires nuclear-cytoplasmic shuttling of p53-MDM2 complexes. It was, however, recently shown that stabilization of p53 by p14\textsuperscript{ARF} occurs independent of the relocalization of MDM2 to the nucleolus (11). This indicates the possibility that the function of ARF may be accomplished by interacting partners other than MDM2. We recently reported that p19\textsuperscript{ARF} binds to Pex19p that sequesters it in the cytoplasm and negatively regulates its p53 activation function (18).

MATERIALS AND METHODS

**Yeast Two-hybrid Screen**—cDNA encoding full-length p19\textsuperscript{ARF} in the yeast expression vector pDDB8 (a kind gift from O. Louvet) was used for screening of the human testis cDNA library in yeast two-hybrid vector pACT2 (Clontech) as described (18). Interactions of two proteins were examined by growth of the yeast transformed by two plasmids on nutrient-deficient (His'/Leu'/Trp/Ade') plates and \(\beta\)-galactosidase reporter activity. The isolated cDNA-derived plasmids were recovered from yeast and sequenced using an ABI sequencer (PerkinElmer Life Sciences).

**Plasmids, Cells, and Transfections**—CARF was cloned into pcDNA3.1/V5, pcDNA4/HisMax (Invitrogen), pEGFPN1 (Clontech), pcQE30 (Qiagen), and pMT-CB6 (19). The integrity of the plasmids was confirmed by sequencing. NIH 3T3 (mouse immortal cells, a kind gift from J. Kato) and U2OS (human osteosarcoma, a kind gift from G. Peters) cells were induced for ARF expression by addition of ZnSO\(_4\) (100 \(\mu\)M) and IPTG (0.01–0.1 mM), respectively (12, 18, 19). Saos-2 (osteosarcoma) and HeLa (cervical carcinoma) cells were also cultured in Dulbecco’s modified Eagle’s minimal essential medium supplemented with 10% fetal bovine serum for staining. Transfections were performed using LipofectAMINE™ PLUS (Invitrogen). Typically, 3 and 10 \(\mu\)g of plasmid DNA were used per 80% confluent 6- and 10-cm dishes, respectively.

**Anti-CARF Antigen and Antibody**—His-tagged CARF protein was expressed in Escherichia coli and purified by its binding to nickel-nitrilotriacetic acid-agarose (Qiagen). Purity of the protein preparations was confirmed by SDS-PAGE and silver staining and was used for rabbit immunizations. An anti-CARF antibody thus obtained was analyzed for its reactivity to CARF by immunoprecipitation and Western blotting.

**Western Analysis**—The protein sample (10–20 \(\mu\)g) separated on a SDS-polyacrylamide gel was electroblotted onto a nylon membrane (Millipore) using a semi-dry transfer blotter (Biorad, Tokyo). Immunoblots were performed with anti-hemagglutinin tag (Santa Cruz), anti-V5 tag (Invitrogen), anti-p53 (Santa Cruz), anti-MDM2 (Santa Cruz), anti-p14ARF (12), and anti-actin (Roche Molecular Biochemicals) antibodies. The immunocomplexes formed were visualized with horseradish peroxidase-conjugated rabbit anti-mouse IgG antibody (ECL kit, Amersham Biosciences).

**Immunostaining**—Cells grown on glass coverslips placed in 35-mm plastic dishes were washed with cold PBS and fixed with 4% formaldehyde.
**Fig. 1.** **A**, lysates (400 µg) from HeLa cells transfected with p19ARF-Myc and CARF-V5 were immunoprecipitated with anti-Myc antibody. The presence of CARF-V5 in ARF immunocomplexes (IC) was examined by Western blotting with anti-V5 antibody (Ab). CARF was co-immunoprecipitated along with p19ARF (lane 3). Vice versa, immunoprecipitation of CARF with anti-V5 antibody pulled down p19ARF (lane 5). Lanes 4 and 6 show control immunoprecipitations with anti-Myc and anti-V5 antibodies, respectively. Lanes 1 and 2 show input (20 µg of lysate) for CARF-V5 and p19ARF-Myc. Note that CARF (580 amino acids) with a predicted molecular mass of 61 kDa runs near to the 84-kDa marker on SDS-PAGE. **B**, characterization of anti-CARF serum. A major band of about 84 kDa was detected on Western blots from lysates of U2OS cells (lane 1). Cells transfected with an expression plasmid encoding CARF-V5 showed the presence of endogenous CARF and a slightly higher sized.
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Fig. 2. CARF accentuates ARF-induced p53 activation. A, NIH 3T3 cells with an inducible expression of ARF were transfected with either empty vector (lanes 1 and 2) or CARF (lanes 3 and 4) and induced for expression of ARF by addition of 100 μM ZnSO4 to the medium. The level of p53 protein and that of its downstream protein, p21WAF1, was enhanced by more than 5-fold by CARF expression in the presence of p19ARF (lanes 2 and 4 of b and c). A mild increase (about 3-fold) in p53 and p21WAF1 was detected in the absence of p19ARF (lanes 1 and 3 of b and c). Expression of p19ARF stabilizes CARF (a, compare lanes 3 and 4), and vice versa expression of CARF stabilized p19ARF (d, compare lanes 2 and 4). B, quantitation of the Western blot; increase (relative units) in the level of proteins (p53 and p21WAF1) normalized against actin is plotted for the presence of ARF, CARF, or ARF and CARF. C, co-existence of ARF and CARF. Three different cell types were stained for endogenous ARF and CARF. In an unsynchronized culture, the cells (marked with white arrows) lacking CARF were also deficient in ARF staining, suggesting the companionship of the two proteins.

CARF-V5 protein (lane 2). The small size proteins in lanes 1 and 2 may be the degraded protein products. CARF-V5 protein was immunoprecipitated with CARF serum but not preserum (compare lanes 3 and 4). The serum IgG (used for immunoprecipitation) cross-reacted to the anti-V5 antibody (smear extending from 50–70 kDa). Anti-p14ARF antibody cross-reacted to a protein of ~70 kDa. a, visualization of CARF-green fluorescent protein (GFP) (a) and p19ARF-Myc (b) in HeLa cells by green fluorescent protein fluorescence and staining with anti-Myc antibody, respectively. Whereas p19ARF localizes predominantly in the nucleolus, CARF was seen in the nucleoplasm as well as in the nucleolus. CARF and p19ARF co-localized only in the periphery (granular region) of nucleoli (seen as a yellow ring in c). NARF (U2OS cells with an inducible expression of p14ARF) cells were transfected with CARF-V5 and were induced for p14ARF expression by 1 mM IPTG overnight. These were doubly stained for CARF (anti-V5 monoclonal antibody) and p14ARF (anti-p14ARF polyclonal serum) (d–f). Co-localization of CARF and ARF was seen in the granular region of the nucleolus of NARF cells. Saos-2 cells were doubly stained for CARF (endogenous) and p14ARF-Myc (exogenous) with a polyclonal CARF antiserum and a monoclonal anti-Myc antibody, respectively. Note the co-localization of the proteins in the granular region of the nucleolus (g–i). HeLa cells stained for endogenous CARF and endogenous ARF also showed co-localization of the proteins in the granular region of the nucleolus (j–l). Taken together, CARF and ARF proteins (tagged exogenous or endogenous) co-localize in the granular region of the nucleolus (a–l). E, a higher magnification image of CARF and ARF showing co-localization of the two proteins in the nucleolus in its periphery (granular region).

In Vivo Co-immunoprecipitation

Immunoassays—U2OS cells were transfected for IPTG-inducible expression of ARF were secondarily transfected with expression plasmid for metal-inducible CARF and puromycin (for puromycin selection) in the ratio of 20:1, respectively. Transfected cells were selected in puromycin (2.5 μg/ml)-supplemented medium for 4 days and were plated (1000 cells per 10-cm dish) in duplicate. Cells were treated with IPTG (1 μg/ml) for ARF expression and ZnSO4 (100 μM) for CARF expression. Cells were maintained until the appearance of colonies with a regular change of medium. Colonies were fixed in methanol, stained with 10% Giemsa solution, photographed, and counted.

CARF RNA Interference (RNAi)—21-Nucleotide RNAs were chemically synthesized using Expedite RNA phosphoramidites and thymidine phosphoramidite. Synthetic oligonucleotides were deprotected and gel-purified. Sequences of two control and two target oligos were 5'-AAAGCACGACUCCUGAGGCCGUT-3', 5'-GCCUCACCGUGACUCGUCCUUT-3' and 5'-CGGAUGUACCCUGCCAGGCAAUT-3', 5'-UCUGGCUCCAGGUACUCCGUT-3', respectively. For annealing of siRNAs, 20 μM of two control or target single strands were incubated in annealing buffer (100 mM potassium acetate, 30 mM HEPES-KOH at pH 7.4, 2 mM magnesium acetate) for 1 min at 90 °C followed by 1 h at 37 °C. Transfections of siRNA duplexes were carried out using Oligofectamine reagent (Invitrogen). Typically, 1–5 μl of the 20 μM duplexes were used per 12-well dish and were assayed after 24–48 h by immunostaining and Western blotting with anti-CARF antibody.

 Colony-forming Assays—U2OS cells stably transfected for IPTG-inducible expression of ARF were secondarily transfected with expression plasmid for metal-inducible CARF and puromycin (for puromycin selection) in the ratio of 20:1, respectively. Transfected cells were selected in puromycin (2.5 μg/ml)-supplemented medium for 4 days and were plated (1000 cells per 10-cm dish) in duplicate. Cells were treated with IPTG (1 μg/ml) for ARF expression and ZnSO4 (100 μM) for CARF expression. Cells were maintained until the appearance of colonies with a regular change of medium. Colonies were fixed in methanol, stained with 10% Giemsa solution, photographed, and counted.
RESULTS AND DISCUSSION

To isolate p19⁰⁰₉₉-ARF-interacting proteins, a Gal4 binding domain (BD)-p19⁰⁰₉₉ fusion protein was used as a bait to screen a human cDNA library cloned into a Gal4 activation domain (AD) yeast two-hybrid plasmid. Two clones, A-1 (Pex19p) (18) and A-10, were strongly positive by His prototrophy and induction of β-galactosidase expression. The nucleotide sequence of A-10 clone (accession number AF 246705, assigned to chromosome 4) matched with the sequence from a cDNA clone (accession number NM-017612; NEDO human cDNA sequencing project). We isolated a full-length cDNA encoding a novel serine-rich (21%) protein consisting of 580 amino acids and named it CARF for a novel protein that collaborates/cooperates with ARF as demonstrated in this study. The amino acid sequence of CARF did not match significantly with any of the protein entries in the database and did not reveal the presence of any known protein motifs. Therefore no hints to its putative function could be obtained. To detect p1⁰⁰⁰⁰₉₉-ARF (ARF) and CARF interactions in mammalian cells, we transfected HeLa cells with expression plasmids encoding CARF-V5 and p1⁰⁰⁰⁰₉₉-Myc proteins and performed in vivo co-immunoprecipitation assays (Fig. 1A). CARF was precipitated with p1⁰⁰⁰⁰₉₉ⁿ, and vice versa, the control immunoprecipitations performed from untransfected cells and control IgG were negative. The results revealed that p1⁰⁰⁰⁰₉₉ⁿ interacts with CARF. Similar co-immunoprecipitation of p1⁰⁴⁰₉₉ was obtained with CARF from cell lysates transfected with CARF-V5 and p1⁰⁴⁰₉₉-Myc (data not shown). To rule out the possibility that the interactions are because of overexpression of the two proteins, we next immunoprecipitated endogenous CARF from NARF cells with a mild level of expression of ARF (U2OS cells that were induced for ARF expression by 0.01 mM IPTG for 12 h). An antibody raised against a full-length recombinant CARF protein and demonstrated to be reactive to CARF by Western blotting and immunoprecipitation (Fig. 1B) was used for immunoprecipitation of endogenous CARF. Immunoprecipitation of endogenous CARF pulled down p1⁰⁴⁰₉₉ (Fig. 1C, level of p1⁰⁴⁰₉₉ protein in one-twentieth of the lysate used for immunoprecipitation is seen in lane 1). These data supported CARF-ARF interactions in vivo. Taken together, it was concluded that CARF interacts with both p1⁰⁰⁰⁰₉₉ and p1⁰⁴⁰₉₉.

To determine whether ARF and CARF proteins co-localize within intact cells, we performed co-immunolocalization studies for exogenous and endogenous proteins (Fig. 1D). In all cell types (HeLa, NIH 3T3, and COS 7) used, p1⁰⁰⁰⁰₉₉ localized mainly in the nucleolus with some diffuse staining in the nu-
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The data were further supported by CARF and ARF staining of unsynchronized cells. In three different cell lines, cells lacking ARF were also deficient in CARF staining (Fig. 2C), suggesting that CARF and ARF are co-regulated. Such co-absence of ARF and CARF was seen in ~70% of three different types of cells examined. Llanos et al. (11) have shown that only a minor fraction of p14ARF is bound to MDM2 and the nucleolar localization of ARF and MDM2 is not essential for the p53 activation function of ARF. We have shown here that CARF is a novel binding partner (collaborator) of ARF; it co-localizes with ARF in the nucleolus, co-regulates with it, and cooperates in its p53 activation function.

To further elucidate the role of CARF for ARF-mediated p53 activation function, we next employed RNA interference (RNAi) in NARF cells (U2OS with inducible expression of p14ARF). The transfection of target siRNA resulted in loss of CARF expression as observed by immunostaining (Fig. 3A, significant decrease in the intensity of CARF staining was observed in 60–70% cells) and Western blotting (Fig. 3, B, compare lanes 1 and 2 with 3–6, and C). As expected, induction of p14ARF caused increased levels of p53 and p21WAF1 (Fig. 3, B, compare lanes 3 and 4 and lanes 5 and 6). In the presence of CARF-siRNA (Fig. 3B, lanes 1 and 2), the p14ARF-induced increase in p53 and p21WAF1 was reduced to about 55%. These data showed that CARF is required for efficient ARF function. Noticeably, ARF levels were also reduced when CARF was targeted (Fig. 3, B and D). These data strongly supported co-regulation of ARF and CARF (also shown in Fig. 2, B and C). Taken together, it was concluded that ARF and CARF are co-regulated and CARF cooperates with ARF in its p53 activation function.

We next examined the biological activity of CARF by the colony-forming assay of U2OS cells (have stably integrated IPTG-inducible p14ARF expression plasmid (12) and metal-inducible expression of CARF along with endogenous CARF) (Fig. 4A). As expected, induction of ARF expression resulted in reduced colony number and size (Fig. 4A, compare a and d with b and c, respectively). Expression of CARF along with ARF caused further reduction (Fig. 4A, compare b and c with e and f, respectively). These results are consistent with the cooperative role of CARF in ARF function and thus assign CARF as a novel regulator of the p19ARF-MDM2-p53 pathway. ARF is an important mediator of cellular senescence and regulates a variety of other p53-dependent or -independent cellular responses (2, 5, 6, 22–24). Intriguingly, CARF was seen to cause some reduction (up to 20–30%) in colony-forming efficiency of cells in the absence of ARF (Fig. 4B). This can be explained, at least in part, by CARF-induced moderate stabilization and activation of p53 in the absence of p19ARF (Fig. 2A) and suggests that CARF may partly function independent of ARF; thus studies on its relation to other members of the ARF-MDM2-p53-p21WAF1 pathway are warranted. Elucidation of mechanism(s) of ARF action, its binding partners, and factors that regulate its activity are of critical importance in our understanding of tumor growth, progression, and therapeutics. We have identified CARF as a novel ARF binding partner. Besides their interactions and co-regulation in vivo, we have demonstrated that CARF cooperates with ARF in its p53 activation function.

Acknowledgments—We thank Emma Duncan and Manami Ohtaka for technical assistance and Gordon Peters for NARF cells and critical reviewing of the manuscript.

REFERENCES
1. Kamijo, T., Zindy, F., Roussel, M. F., Quelle, D. E., Downing, J. R., Ashmun, R. A., Grosveld, G., and Sherr, C. J. (1997) Cell 91, 649–659
2. Kamijo, T., van de Kamp, E., Chong, M. J., Zindy, F., Diehl, J. A., Sherr, C. J., and McKinnon, P. J. (1999) Cancer Res. 59, 2464–2469
3. Quelle, D. E., Zindy, F., Ashmun, R. A., and Sherr, C. J. (1995) Cell 83, 993–1000
4. Serrano, M., Lee, H., Chin, L., Cordon-Cardo, C., Beach, D., and DePinho, R. A. (1996) Cell 85, 27–37
5. Sharpless, N. E., and DePinho, R. A. (1999) Curr. Opin. Genet. Dev. 9, 22–30
6. Carnero, A., Hudson, J. D., Price, C. M., and Beach, D. H. (2000) Nat. Cell. Biol. 2, 148–155
7. Jacobs, J. J., Scheijen, B., Voneken, J. W., Kieboom, K., Berns, A., and van Lohuizen, M. (1999) Genes Dev. 13, 2678–2690
8. Vogt, M., Haggblom, C., Yeargin, J., Christiaensen-Weber, T., and Haas, M. (1998) Cell Growth, and Differ. 9, 139–146
9. Serrano, M. (1997) Exp. Cell Res. 237, 7–13
10. Honda, R., and Yasuda, H. (1999) EMBO J. 18, 22–27
11. Llanos, S., Clark, P. A., Rowe, J., and Peters, G. (2001) Nat. Cell. Biol. 3, 445–452
12. Stott, F. J., Bates, S., James, M. C., McConnell, B. B., Starborg, M., Brookes, S., Palmero, I., Ryan, K., Hara, E., Vousden, K. H., and Peters, G. (1998) EMBO J. 17, 5001–5014
13. Tao, W., and Levine, A. J. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 6937–6941
14. Weber, J. D., Taylor, L. J., Roussel, M. F., Sherr, C. J., and Bar-Sagi, D. (1999) Nat. Cell. Biol. 1, 20–26
15. Weber, J. D., Kuo, M. L., Bothner, B., DiGiammarino, E. L., Kriwacki, R. W., Roussel, M. F., and Sherr, C. J. (2000) Mol. Cell. Biol. 20, 2517–2528
16. Zhang, Y., Xiong, Y., and Yarbrough, W. G. (1998) Cell 92, 725–734
17. James, M. C., and Peters, G. (2000) Prog. Cell Cycle Res. 4, 71–81
18. Sugihara, T., Kaul, S. C., Kate, J., Reddel, R. R., Nomura, H., and Wadhwa, R. (2001) J. Biol. Chem. 276, 18649–18652
19. Kurokawa, R., Tanaka, T., and Kato, J. (1999) Oncogene 18, 2718–2727
20. Kamijo, T., Weber, J. D., Zambetti, G., Zindy, F., Roussel, M. F., and Sherr, C. J. (1999) Proc. Natl. Acad. Sci. U. S. A. 95, 8292–8297
21. Kamijo, T., Bodner, S., van de Kamp, E., Randle, D. H., and Sherr, C. J. (1999) Cancer Res. 59, 2217–2222
22. Serrano, M., Lin, A. W., McCurrach, M. E., Beach, D., and Lowe, S. W. (1997) Cell 88, 593–602
23. Dimri, G. P., Itahana, K., Acosta, M., and Campisi, J. (2000) Mol. Cell. Biol. 20, 273–285
24. Tolbert, D., Lu, X., Yin, C., Tantama, M., and Van Dyke, T. (2002) Mol. Cell. Biol. 22, 370–377