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The ARF tumor suppressor inhibits BCL6-mediated transcriptional repression

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Abstract

The *ARF* tumor suppressor gene antagonizes generation of various tumors. ARF-mediated tumor suppression occurs in a p53-independent manner as well as in a p53-dependent manner. We here demonstrate that BCL6 is a target of the ARF tumor suppressor. Either mouse $p19^{ARF}$ or human $p14^{ARF}$ binds to BCL6 and downregulates BCL6-induced transcriptional repression. ARF-mediated downregulation of the BCL6 activity may account in part for ARF-mediated tumor suppression.

Keywords: ARF; $p19^{ARF}$; $p14^{ARF}$; BCL6; Tumor suppressor

The *ARF* tumor suppressor gene antagonizes the occurrence of various tumors [1,2]. ARF upregulates the p53 tumor-suppressing activity by interacting with Mdm2 and increasing protein expression of p53. In addition to such p53-dependent tumor-suppressive activity, it has been established that ARF has the p53-independent tumor-suppressing activity [3]. In agreement, enforced expression in vitro of the ARF tumor suppressor induces cell cycle arrest [4] as well as apoptosis [5–8] not only in p53-intact cells but also in p53-deficient cells.

The molecular mechanism underlying p53-independent apoptosis mediated by the *ARF* gene has been partially elucidated. Using mouse embryonic fibroblasts functionally deficient in p53 and Bax or Bak, it has been shown that ARF-mediated p53-independent apoptosis occurs largely via Bax [9]. Another study has indicated that upregulation of Bim-L and Bax expression levels in the mitochondria-containing fraction seems to contribute to p53-independent ARF-mediated apoptosis [8]. It has also been pointed out that overexpression of ARF induced expression of various growth-inhibiting genes such as four members of the B cell translocation gene family (Btg1, Btg2, Btg3, and Tob1), whose enforced expression induces cell cycle retardation [10].

Another important clue to elucidate the p53-independent pathway has been indicated by a series of studies regarding ARF-binding proteins such as E2F [11,12], the phosphatase-binding protein spinophilin [13], the peroxisomal protein Pex19P [14], topoisomerase I [15], HIF-1 [16], cyclinG1 [17], and NF-κB [18]. However, biological outcomes of these interactions have not well characterized.

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BCL6, a sequence-specific transcriptional repressor of various genes, is physiologically essential for B cell development in the germinal center [19–21]. The *BCL6* gene has been originally identified as the gene frequently rearranged and overexpressed in B cell lymphoma. Most recently, it has been shown that constitutive overexpression of BCL6 in lymphocytes resulted in development of B cell lymphoma in a minor fraction of transgenic mice [22]. Accordingly, BCL6 has been regarded as an oncogene contributing to development of B cell lymphoma ([23] for review). However, some in vitro studies have indicated that ectopic expression of BCL6 induces apoptosis and cell cycle arrest in certain cells, suggesting that BCL6 becomes a tumor suppressor in certain situations, indicating that the roles of BCL6 in generation of lymphomas are still elusive.

We here demonstrate that both mouse $p19^{ARF}$ and human $p14^{ARF}$ associate with BCL6. By binding to BCL6, they inhibit BCL6-mediated transcriptional repression. We speculate that the ARF tumor suppressors may exert their anti-tumor activities in part by this molecular interaction.

Materials and methods

Cell culture and transfection. U2OS cells (p53-intact) and COS7 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 2 mM glutamine. Primary p53/Mdm2-null mouse embryonal fibroblasts (MEFs) were kind gifts from Dr. C.J. Sherr and Dr. M.F. Roussel (St. Jude Children's Res. Hosp., TN).

Transient transfection to COS7 cells and U2OS cells was performed with lipofectAMINE PLUS reagents according to the manufacturer's instructions (Gibco-BRL, MD) [24]. COS7 cells (80–100% confluency) in 60-mm dishes were incubated for 3 h with pre-complexed DNAs and the lipofectAMINE PLUS reagents. Unless specified, 2–3 μg of each DNA, 10–12 μl PLUS, and 10–12 μl lipofectAMINE reagents were used for each dish. Transfection to cells on the 48-well dishes was performed using 0.75 μl PLUS reagent and 0.4 μl lipofectAMINE per transfection.

Plasmids and adenoviral vectors. Backbone vectors, pMF and pEBG, were used for mammalian expression of FLAG-tagged proteins and GST-tagged proteins [24]. pEF-myc-BCL6 was kindly provided by Dr. V. Bardwell (Minneapolis, MN). To construct GST-BCL6-ΔC, we inserted the 1.8 kb *Bam*HI–*Eco*RI BCL6 cDNA fragment into the pEBG vector. The pGL2-control plasmid containing two BCL6-binding sites upstream SV40 promoter (2× BS-Luc) was kindly provided by Dr. M. Hatano and Dr. T. Tokuhisa (Chiba, Chiba). cDNAs for BCL6-RD, BCL6-MD, and BCL6-DD were PCR-amplified using sense primers (5′-AGCGAATTCGGATCCATGGCCTCGCCGGCTGAT, 5′-AGCGAATTCGGATCCATGACACCGCCAGCCTCTTAT, and 5′-AGCGAATTCGGATCCACACCGCCAGCCTCTTAT) and antisense primers (5′-AGCCTCAGAATCGATTCATTCACGAGGAGGCTTGAT, 5′-AGCCTCGAGATCGATTCAGGACTGGAGGTCAAGGTT, and 5′-AGTCTCGAGATCGATTCAGCAGGCTTTGGGGAG), respectively.

The system of a replication-deficient adenoviral vector, described in detail [24], was purchased from TaKaRa (Shiga, Japan). An adenovirus cre/loxP-regulated expression vector was also purchased from TaKaRa. The adenoviruses encoding cre-$p19^{ARF}$ viruses and its various derivative viruses were previously described [7].

Adenoviral-vector mediated expression. All viruses were grown in HEK293 cells and purified by double CsCl gradient ultracentrifugation [24]. Infection was carried out by adding recombinant adenoviruses to serum-containing media as described. Unless specified, cells were incubated with virus-containing media at the indicated multiplicity of infection (moi) at 37 °C for 60 min.

Antibodies. Rabbit polyclonal antibodies to BCL6 were previously described [25,26]. A rabbit polyclonal antibody (C-19) and a mouse monoclonal antibody to BCL6 (D-8) were purchased from Santa Cruz Biotech (Santa Cruz, CA). Mouse monoclonal antibodies to GST (B-14) were purchased from Santa Cruz Biotech. The monoclonal antibodies to FLAG (M2) were from Oncogene Science (Cambridge, MA) and Eastman Kodak (Kingsport, TN). A rabbit polyclonal antibody to $p19^{ARF}$ (Ab80) was purchased from Abcam (Cambridge, UK).

Immunoprecipitation, pull-down assays, and immunoblotting. Cells were suspended in a NP40 lysis buffer (50 mM Hepes, 150 mM NaCl, 1 mM EDTA, 1 mM DTT, and 0.5% Nonidet P-40) containing 2.5 μg/ml leupeptin, 5 μg/ml aprotinin, and 0.2 mM phenylmethylsulfonyl fluoride (PMSF) and sonicated at 4 °C. To reduce backgrounds for immunoprecipitation, supernatants from cell lysates were pre-incubated with 10 μl of non-immune rabbit serum and 20 μl of 1:1 slurry of protein G–Sepharose FF (Pharmacia) for 1–2 h. The cleared supernatants were then incubated for 2 h with indicated antibodies and precipitated for 1 h with 20 μl of 1:1 slurry of protein G–Sepharose FF at 4 °C. The washed immunoprecipitates were used for further experiments. To perform pull-down assays, the cleared cell lysates were incubated with 20 μl of 1:1 slurry of glutathione–Sepharose at 4 °C for 2 h. GST or GST-tagged proteins in the cell lysates were adsorbed onto glutathione–Sepharose as precipitates [24]. Immunoblotted signals were visualized with an ECL detection kit from Amersham–Pharmacia Biotech (Uppsala, Sweden).

Immunocytochemistry. U2OS cells (5×10^4), seeded onto six-well plates and infected with adenoviruses, were fixed at 24 h after infection with 100% ethanol for 20 min at room temperature, rinsed for 45 min with phosphate-buffered saline (PBS), and then stained with the first antibodies for 60 min at 37 °C. After being washed for 45 min with PBS, cells were stained with FITC-conjugated goat anti-rabbit IgG (Vector Lab., CA) or Texas-red-conjugated goat anti-mouse IgG (Vector Lab.) for 60 min at 37 °C. Before detection, they were extensively washed for 45 min with PBS. Fluorescence signals were detected with a laser scanning, confocal microscope LSM (Carl Zeiss, Germany).

Luciferase assays. COS7 cells (4×10^4/well) or U2OS cells ($1.5–3.0 \times 10^4$/well), seeded onto 48-well plates, were transfected with 0.025 μg of 2× BS-Luc as a reporter together and 0.05–0.1 μg of the pEF plasmid or pEF-myc-BCL6 together with indicated amounts of pMF-$p14^{ARF}$ or pMF-$p19^{ARF}$. To keep the total amount of each plasmid constant, we added the appropriate amounts of backbone plasmids. In all experiments, pRL-TK *Renilla* luciferase vectors from Promega (Madison, WI) were co-transfected to monitor transfection efficiency. At 33 h after transfection, luciferase assays were performed with Dual-Luciferase Reporter Assay System (Promega). The firefly luciferase activities were adjusted by transfection efficiency indicated by the *Renilla* luciferase activities. By monitoring and comparing the *Renilla* luciferase activities at several time points after transfection, we concluded that either BCL6- and ARF-mediated cell death does not occur at least until 33 h after transfection (data not shown).

Results

Physical interaction between the ARF tumor suppressor and BCL6

To see the association of $p19^{ARF}$ (the mouse ARF) or $p14^{ARF}$ (the human ARF) with BCL6 in cells, we first

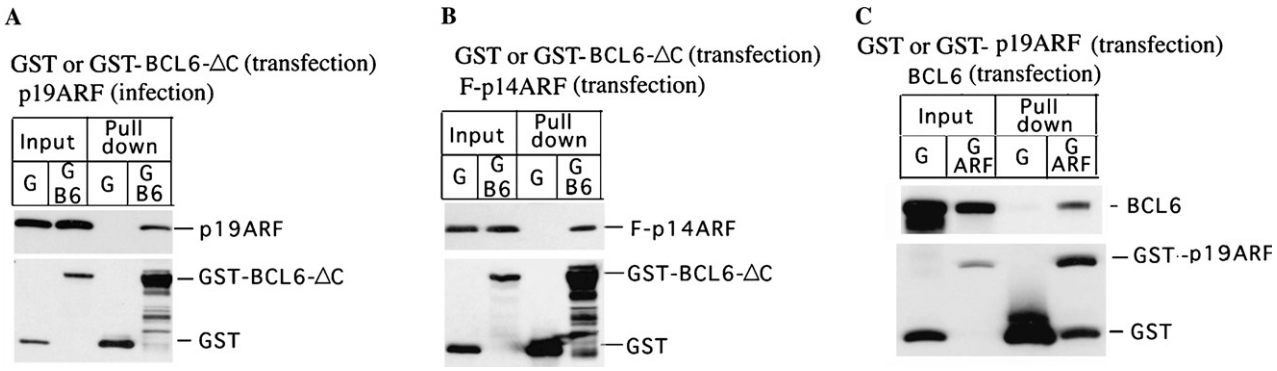

Fig. 1. Association between ARF and BCL6. (A) COS7 cells (5×10^5), transfected with pEBG or pEBG-BCL6-ΔC, were subsequently infected with the cre-p19^{ARF} virus at a moi of 80 and the cre-recombinase virus at a moi of 40. (B) COS7 cells (5×10^5) were co-transfected with pEBG or pEBG-BCL6-ΔC together with pMF-p14^{ARF}. (C) COS7 cells (5×10^5) were co-transfected with pEBG or pEBG-p19^{ARF} together with pEF-BCL6. At 48 h after transfection, cells were harvested for pull-down assays with glutathione beads. One-fiftieth the amount of lysates was used for lanes of "input." The membrane was used for immunoblotting with antibodies to p19^{ARF}, FLAG, BCL6, or GST as indicated. G, GST; B6, BCL6-ΔC.

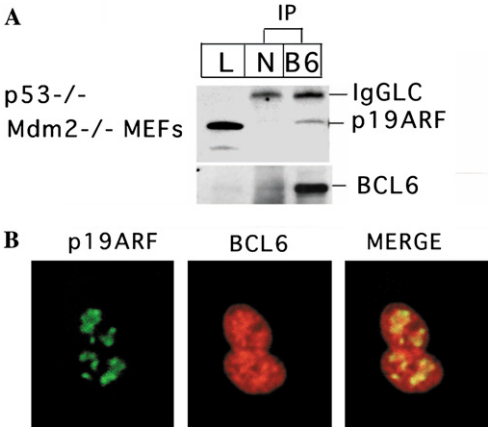

Fig. 2. (A) Endogenous p19^{ARF} associates with endogenous BCL6. Lysates from MEFs (4×10^6) were immunoprecipitated with indicated antibodies. For the "L" lane, one-fiftieth the amount of cell lysates used for immunoprecipitation was applied. " N" and "B6" indicate non-immune rabbit sera and anti-BCL6 antibodies, respectively, that were used for immunoprecipitation. The same membrane was used for sequential immunoblotting with antibodies to p19^{ARF} and BCL6. (B) Subcellular localization of p19^{ARF} and BCL6. U2OS cells (5×10^4) were infected with the BCL6 virus at a moi of 200 in association with the cre-p19^{ARF} virus at a moi of 200 and the cre-recombinase virus at a moi of 40. At 24 h after infection, cells were fixed and stained with indicated antibodies.

performed pull-down analysis. After overexpressing GST-tagged BCL6-ΔC, deficient in the C-terminal 110 amino acids of BCL6, together with p19^{ARF} or FLAG-p14^{ARF} in COS7 cells and performing pull-down assays with glutathione beads, we found that GST-BCL6-ΔC co-precipitated with p19^{ARF} or FLAG-p14^{ARF} in COS7 cells (Figs. 1A and B). Reciprocally, another pull-down assay indicated that GST-p19^{ARF} co-precipitated with BCL6 (Fig. 1C). Furthermore, we performed co-immunoprecipitation analysis using p53/Mdm2-null mouse embryonal fibroblasts, and found that endogenous p19^{ARF} was co-immunoprecipitated with endogenous BCL6 (Fig. 2A), supporting the notion that the ARF tumor suppressor biologically interacts with BCL6, a putative oncogene involved in the development of B cell lymphoma.

It has been established that the ARF tumor suppressor mainly localizes in the nucleolus while ARF localizes in the non-nucleolar area in the nucleus [27]. BCL6, a sequence-specific transcription factor, displays speckled distribution in the nucleus [28]. Immunofluorescence analysis was performed to see co-localization of both proteins (Fig. 2B). As expected, p19^{ARF} showed its main distribution in the nucleolus while BCL6 was present all over the nucleus with speckled distribution. Partially overlapped distribution of BCL6 and p19^{ARF} was seen in the merge figure (Fig. 2B, merge).

Identification of domains of BCL6 and p19^{ARF} involved in mutual interaction

We further asked which regions of BCL6 and ARF are involved in interaction between BCL6 and ARF. Adenoviruses harboring N-terminally FLAG-tagged p19^{ARF} mutants consisting of FLAG-p19^{ARF} 1–37, FLAG-p19^{ARF} 37–169, FLAG-p19^{ARF} 68–129, or FLAG-p19^{ARF} 130–169 have been made using a cre/loxP-regulated adenovirus system [27]. We co-expressed one of these mutants together with pEBG-BCL6-ΔC encoding GST-BCL6-ΔC and then performed a pull-down assay (Fig. 3A). As shown in Fig. 3A, both FLAG-p19^{ARF} 1–37 and FLAG-p19^{ARF} 37–169 bind to BCL6-ΔC. However, it is apparent that FLAG-p19^{ARF} 68–129, the middle region of p19^{ARF}, hardly binds to BCL6 while FLAG-p19^{ARF} 130–169, the C-terminal rodent-specific region, weakly binds to BCL6. We accordingly concluded that there are at least two domains of p19^{ARF} interacting with BCL6, which correspond to the N-terminal domain including p19^{ARF} 1–37 and the C-terminal domain including p19^{ARF} 130–169 (Fig. 3A,

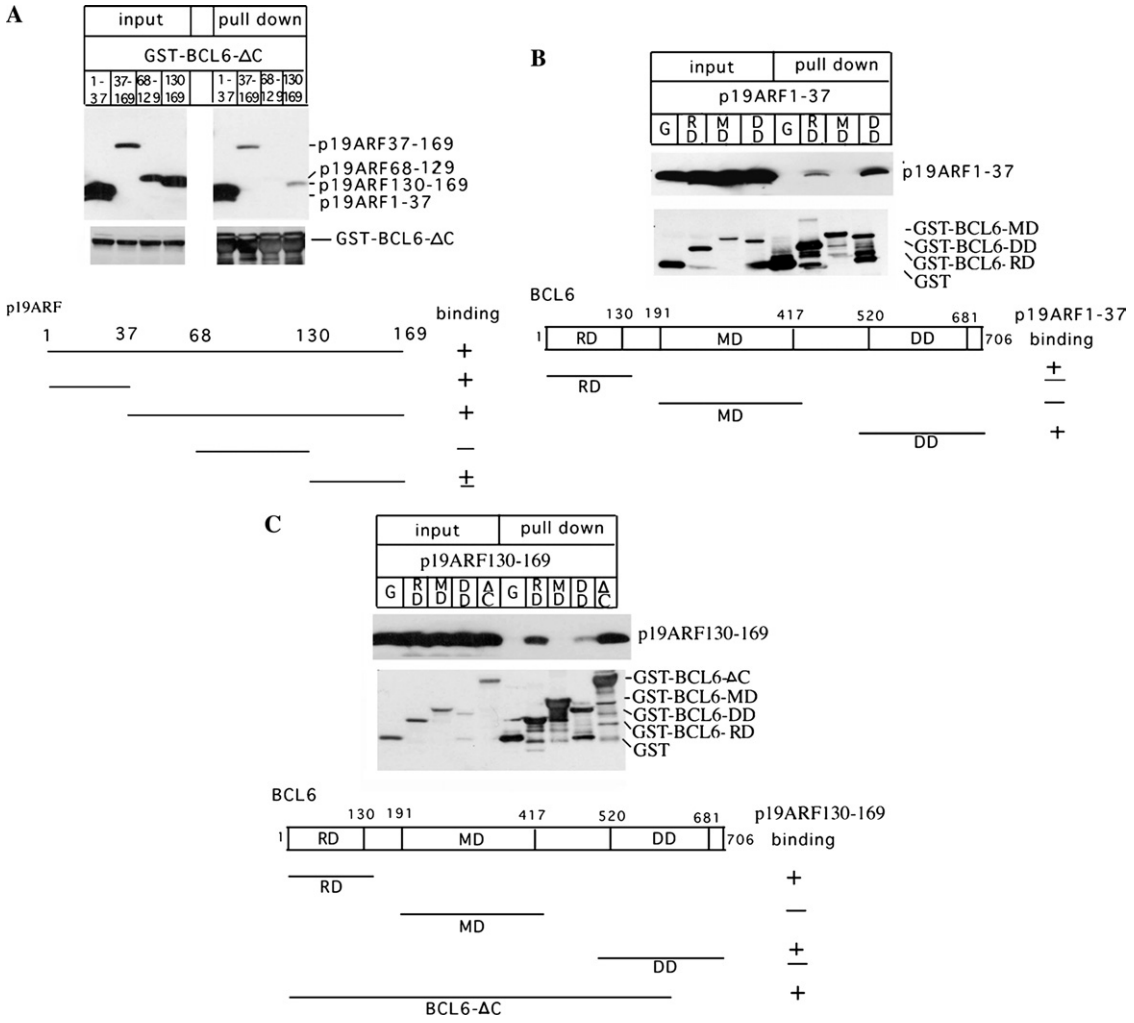

Fig. 3. Identification of domains of BCL6 and ARF involved in mutual interaction. (A) COS7 cells (6×10^5), transfected with pEBG or pEBG-BCL6-ΔC, were subsequently infected with cre/loxP-regulated adenoviruses encoding indicated FLAG-tagged deletion mutants of $p19^{ARF}$ in association with cre-recombinase viruses at a moi of 40. At 48 h after transfection, cells were harvested for the pull-down assays. Immunoblot analysis was performed with antibodies to FLAG (ARF mutants) and GST. One-fiftieth the amount of lysates was used for lanes of "input." In the bottom panel, schematic illustration of various $p19^{ARF}$ deletion mutants is shown. (B) COS7 cells (6×10^5) were transfected with pEBG, pEBG-BCL6-RD, pEBG-BCL6-MD, or pEBG-BCL6-DD. After transfection, cells were infected with the adenovirus encoding FLAG-$p19^{ARF}$ 1–37 at a moi of 400 in association with the cre-recombinase virus at a moi of 40. At 48 h after transfection, cells were harvested for the pull-down assay. Immunoblot analysis was performed with antibodies to FLAG ($p19^{ARF}$ 1–37) and GST. One-fiftieth the amount of lysates was used for lanes of "input." In the bottom panel, schematic illustration of various BCL6 deletion mutants is shown. G, GST; RD, the repression domain; MD, the middle domain; and DD, the zinc finger DNA-binding domain. (C) COS7 cells (6×10^5), were transfected with pEBG, pEBG-BCL6-RD, pEBG-BCL6-MD, pEBG-BCL6-DD, or pEBG-BCL6-ΔC. After transfection, cells were infected with adenovirus encoding FLAG-$p19^{ARF}$ 130–169 at a moi of 40 in association with cre-recombinase-encoding viruses at a moi of 40. At 48 h after transfection, cells were harvested for the pull-down assay. Immunoblot analysis was performed with antibodies to FLAG ($p19^{ARF}$ 130–169) and GST. One-fiftieth the amount of lysates was used for lanes of "input." In the bottom panel, schematic illustration of various BCL6 deletion mutants is shown. G, GST; RD, the repression domain; MD, the middle domain; and DD, the DNA-binding zinc finger domain.

bottom), although we did not exclude a possibility that there may be another region in the middle part of $p19^{ARF}$ involved in their interaction.

To address the question which portions of BCL6 interact with $p19^{ARF}$ 1–37, systematic deletion mutants of BCL6 were constructed (Fig. 3B, bottom). BCL6 is composed of the transcription-repressive domain (RD) corresponding to the POZ domain, the middle domain (MD), and the DNA-binding zinc finger domain (DD). A pull-down experiment with glutathione beads showed that FLAG-$p19^{ARF}$ 1–37 was co-precipitated with GST-BCL6-DD, and mildly with GST-BCL6-RD, but not with GST-BCL6-MD (Fig. 3B, top). The C-terminal domain corresponding to $p19^{ARF}$ 130–169 is missing from human $p14^{ARF}$ and is therefore unique for rodent *ARF* genes [7]. We further asked which domains of BCL6

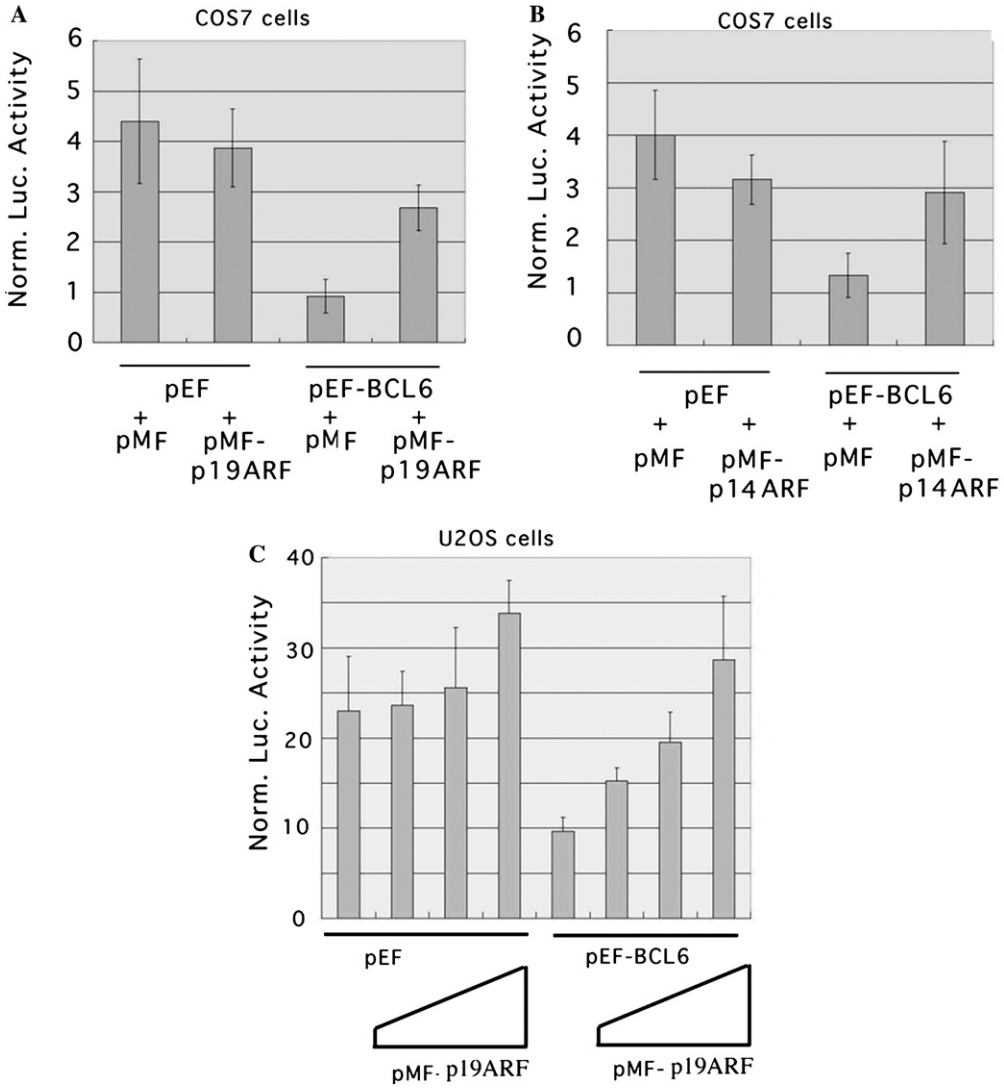

Fig. 4. (A,B) ARF attenuates BCL6-mediated repression. COS7 cells were co-transfected with 0.1 μg of pEF or pEF-myc-BCL6 together with 0.1 μg of the pMF vector, pMF-p19^{ARF} (A) or pMF-p14^{ARF} (B). 0.025 μg of the reporter plasmid, 2× BS-Luc, was co-transfected. 0.025 μg of the TK-*Renilla* luciferase plasmid was co-transfected to monitor transfection efficiency. Luciferase activities were normalized by transfection efficiency. All experiments were performed with $N = 3$. (C) ARF attenuates BCL6-mediated repression in a dose-dependent manner. U2OS cells were co-transfected with 0.05 μg of the pEF plasmid or pEF-myc-BCL6 together with increasing amounts of pMF-p19ARF (0, 0.2, 0.4, and 0.8 μg). To keep the total amount of pMF plasmids constant, appropriate amounts of pMF backbone vectors were added for transfection. 0.025 μg of the reporter plasmid, 2× BS-Luc, was co-transfected. 0.025 μg of the TK-*Renilla* luciferase plasmid was co-transfected to monitor transfection efficiency. Luciferase activities were normalized by transfection efficiency. All experiments were performed with $N = 3$.

are involved in interaction with p19^{ARF} 130–169. A pull-down assay showed that the RD domain and the DD domain interact with p19^{ARF} 130–169 (Fig. 3C).

ARF downregulates BCL6-mediated transcriptional repression

We then asked whether ARF inhibits the BCL6 activity. To examine this possibility, we performed luciferase assays using the pGL2-control plasmid containing 2× BCL6-binding consensus nucleotides upstream of the SV40 promoter (2× BS-Luc) as a reporter. As shown in Figs. 4A and B, expression of BCL6 downregulated the promoter activity of the 2× BS-Luc plasmid possibly by binding to its BCL6-binding consensus nucleotide sequence in COS7 cells. Such BCL6-mediated downregulation of the promoter activity was markedly attenuated by co-expression of either p19^{ARF} or p14^{ARF} (Figs. 4A and B). To confirm these results, we further performed a similar experiment using U2OS cells. In this case, we co-transfected various amounts of the p19^{ARF}-encoding vector together with the BCL6-encoding vector (Fig. 4C). As expected, BCL6-mediated transcriptional repression of the promoter activity was markedly attenuated by co-expression of p19^{ARF} in a dose-responsive fashion even in U2OS cells.

Discussion

Cell cycle arrest and cell death are two major cellular phenotypes directly linked to tumor suppression. The ARF tumor suppressor partially exerts its tumor-suppressive function by interacting with Mdm2 and upregulating p53 activity [1,2]. Upregulation of p53 activities results in cell cycle arrest and cell death. Multiple physical interactors with ARF other than Mdm2 have been identified [11–18]. Among them, E2F seems to be directly linked to cell growth. We here showed that co-expression of ARF markedly downregulates the BCL6-mediated transcriptional repression, suggesting that BCL6 is another putative effector of ARF-mediated tumor suppression.

It remains almost completely unknown how ARF downergulates the BCL6 transcription-repressing activity. ARF does not change the expression level of BCL6 (H.S. and M.M. unpublished observation). One possible mechanism for ARF-mediated downregulation of the BCL6 activity is that ARF inhibits association between BCL6 and the target DNA sequences by binding to BCL6. Gel-retardation assays have indicated, however, that addition of ARF proteins did not reduce association between BCL6 and the target DNA sequence (H.S. and M.M. unpublished observation), ruling out this possibility. Thus, the precise molecular mechanism underlying ARF-mediated suppression of the BCL6 activity still remains to be elucidated.

BCL6 has been considered an oncogene in B cell lymphoma although the manner of its involvement in tumor promotion has not been completely elucidated [23]. Clinical observation and a line of in vitro studies strongly support the notion that it is an oncogene [29–34] while some in vitro studies [25,35–37] suggest that BCL6 has growth-retardation activities when it is ectopically expressed, indicating that BCL6 may be a tumor suppressor in some situations. Disruption of the ARF tumor suppressor leads to progression of a variety of tumors in mice [2], proving that the *ARF* gene is a potent tumor suppressor. The fact that the ARF tumor suppressor inhibits the activity of BCL6 as shown in this study supports the notion that BCL6 is an oncogene. This notion has also been supported by the finding that enforced expression of BCL6 antagonizes cellular senescence of mouse embryonic fibroblasts as well as the germinal center B cells mediated by the ARF tumor suppressor and p53 [38].

In summary, we provide evidence that BCL6 is a putative downstream effector in ARF-induced p53-independent tumor suppression. This finding leads us to conclude that further examination of BCL6 function is required from the standpoint of cell cycle regulation, apoptosis, and tumor formation in order to further understand the function of the ARF tumor suppressor.

Acknowledgments

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