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BRG-1 is required for RB-mediated cell cycle arrest

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The antiproliferative action of the retinoblastoma tumor suppressor protein, RB, is disrupted in the majority of human cancers. Disruption of RB activity occurs through several disparate mechanisms, including viral oncoprotein binding, deregulated RB phosphorylation, and mutation of the RB gene. Here we report disruption of RB-signaling in tumor cells through loss of a critical cooperating factor. We have previously reported that C33A cells fail to undergo cell cycle inhibition in the presence of constitutively active RB (PSM-RB). To determine how C33A cells evade RB-mediated arrest, cell fusion experiments were performed with RB-sensitive cells. The resulting fusions were arrested by PSM-RB, indicating that C33A cells lack a factor required for RB-mediated cell cycle inhibition. C33A cells are deficient in BRG-1, a SWI/SNF family member known to stimulate RB activity. Consistent with BRG-1 deficiency underlying resistance to RB-mediated arrest, we identified two other BRG-1-deficient cell lines (SW13 and PANC-1) and demonstrate that these tumor lines are also resistant to cell cycle inhibition by PSM-RB and p16ink4a, which activates endogenous RB. In cell lines lacking BRG-1, we noted a profound defect in RB-mediated repression of the cyclin A promoter. This deficiency in RB-mediated transcriptional repression and cell cycle inhibition was rescued through ectopic coexpression of BRG-1. We also demonstrate that 3T3-derived cells, which inducibly express a dominant-negative BRG-1, arrest by PSM-RB and p16ink4a in the absence of dominant-negative BRG-1 expression; however, cell cycle arrest was abrogated on induction of dominant-negative BRG-1. These findings demonstrate that BRG-1 loss renders cells resistant to RB-mediated cell cycle progression, and that disruption of RB signaling through loss of cooperating factors occurs in cancer cells.

cyclins | Cdk | SWI/SNF

The retinoblastoma tumor suppressor protein (RB) is a critical regulator of cell cycle progression that is functionally inactivated in the majority of human tumors (1–8). RB functions as a protein-binding protein, binding to greater than 50 identified cellular proteins. However, the requirement of these proteins for RB-mediated cell cycle inhibition is largely unknown. Overall, RB-assembled protein complexes lead to the repression of transcription, and this function of RB is critical for cell cycle regulation. The principal target of RB is believed to be the E2F family of transcriptional activators (6, 9–11). E2F controls the expression of numerous genes directly involved in cell cycle progression or in metabolic processes coupled to DNA replication (6, 9–11). RB binding converts E2F from a transcriptional activator to a repressor through a mechanism that involves the recruitment of histone deacetylases (12, 13). RB also mediates the repression of other gene products, such as cyclin A, through complicated mechanisms that are not clearly understood (14).

In response to mitogenic signaling, RB is phosphorylated in mid-G_1 by Cdk4/cyclin D complexes (1–4). This initial phosphorylation contributes to the disruption of RB protein-binding activity, thus alleviating RB-mediated transcriptional repression (15). RB is the only relevant target for Cdk4/cyclin D, as inhibition of Cdk4 results in cell cycle arrest only in those cells that express a functional RB protein (16–18). Later in G_1, Cdk2/cyclin E, and cyclin A complexes also phosphorylate RB (1–4). However, unlike Cdk4/cyclin D, Cdk2 complexes are required for cell cycle progression in the absence of RB (19, 20). Together, Cdk4 and Cdk2 complexes are required and are rate limiting for cell cycle progression (20).

The mechanism through which RB acts on the cell cycle has been difficult to address, because it is rapidly phosphorylated/inactivated by endogenous Cdk4 and Cdk2 activities (21–24). To circumvent this difficulty, we generated phosphorylation site mutants of RB (PSM-RB), which render it insensitive to Cdk-mediated inactivation (22). Unlike wild-type RB, PSM-RB overexpression arrests the vast majority of tumor cell lines studied, confirming that RB phosphorylation is generally required for progression through the cell cycle (25). Typically, this RB-mediated cell cycle arrest involves the attenuation of cyclin A expression and Cdk2-associated kinase activity, whereas Cdk4, cyclin D1, and cyclin E are unaffected (14, 18, 23). The importance of cyclin A as a target of RB is exemplified by the observation that ectopic cyclin A expression overcomes PSM-RB-mediated cell cycle arrest (14, 26).

Surprisingly, we identified a cell line, C33A, which was resistant to PSM-RB (25). Therefore, this cell line can progress through the cell cycle in the absence of RB phosphorylation/inactivation. We hypothesized that C33A cells either harbor a dominant activity that inactivates RB or lack a cooperating recessive activity required for RB function. Although there are numerous proteins that disrupt RB function (SV40 T-antigen, adenovirus E1A, E2F-1, cyclin E), few have been shown to cooperate with RB (1–4). Prior studies demonstrated that BRG-1, a member of the SWI/SNF chromatin remodeling complex, interacts with RB, and ectopic expression of BRG-1 was shown to cause growth inhibition dependent on interaction with RB (27–32). Furthermore, it was shown that expression of BRG-1 enhances RB-mediated inhibition of E2F transcription (31). These studies suggested that overproduction of BRG-1 can cooperate with RB. Here, we report that BRG-1 is in fact required for RB-mediated signaling to critical downstream effectors and subsequent cell cycle arrest. Moreover, we demonstrate that loss of BRG-1 function in tumor cells renders them resistant to the antiproliferative activity of RB, thus revealing a new mechanism by which tumor cells attain growth advantage.

Materials and Methods

Cells, Plasmids, and Transfection. C33A, SAOS-2, U2OS, SW13, PC3, and PANC-1 cells were cultured as previously described (25). B05–1 cells were grown in DMEM supplemented with 10% heat inactivated calf serum, 100 units/ml penicillin–streptomycin, and 2 mM L-glutamine either in the presence or absence of

Abbreviation: β-gal, β-galactosidase.

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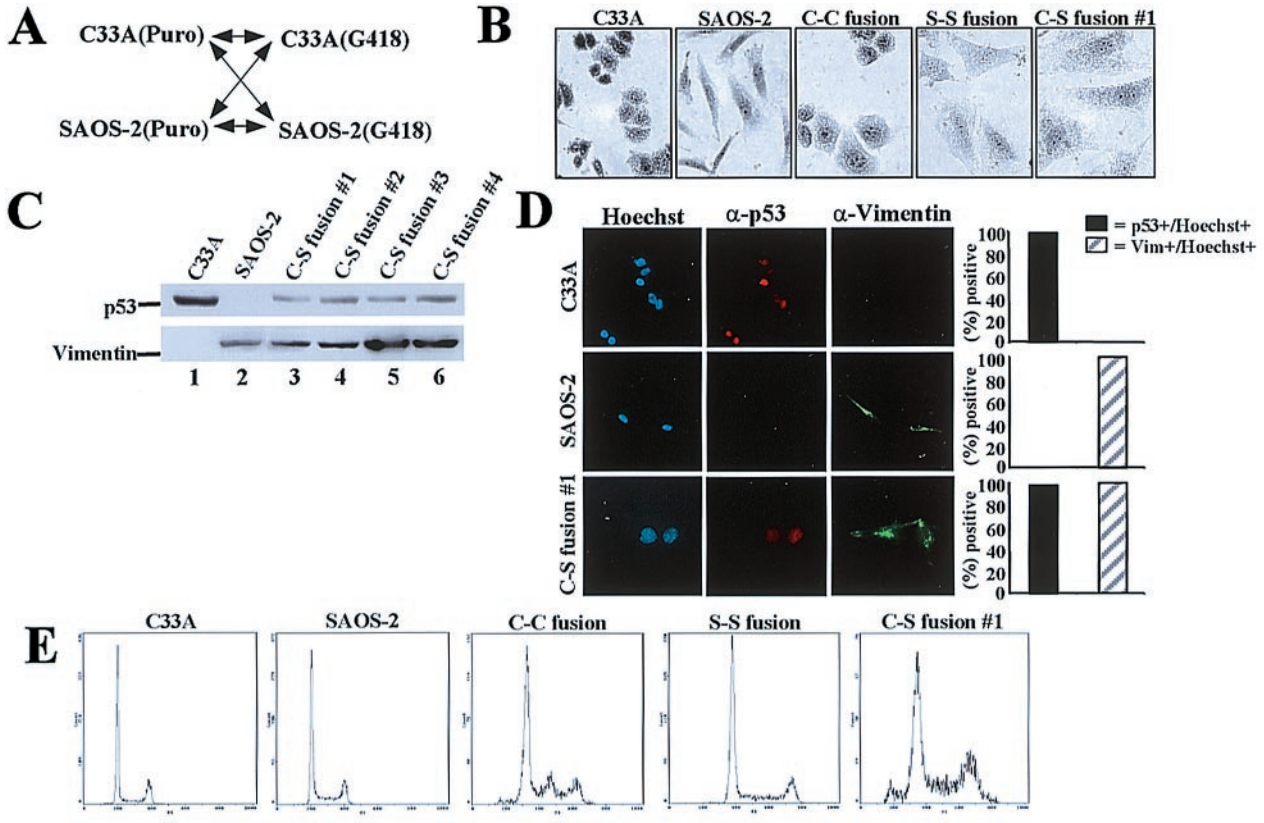

Fig. 1. Fusion of RB-resistant to RB-sensitive cells. (*A*) Cell fusion schema. (*B*) Parental C33A and SAOS-2 cells, as well as the C-C, S-S, and C-S fusions were fixed and stained with Giemsa. (*C*) The fused and nonfused parental cells were lysed, and equal total protein was resolved by SDS/PAGE then immunoblotted for p53 and vimentin. (*D*) Fused and nonfused parental cells were fixed and then coimmunostained for vimentin and p53. (*E*) Proliferating fused and nonfused cells were trypsinized, fixed, and then stained with propidium iodide. The DNA content of the stained cells was then determined by flow cytometry.

2 μg/ml tetracycline (33). To generate antibiotic resistant lines, C33A and SAOS-2 cells were transfected with pBabe-Puro or CMV-NEO, as previously described (34). Selection was carried out with 2.5 μg/ml puromycin or 600 μg/ml G418. The following plasmids, CMV-NEO, −608CycALuc, E2F/Luc, pH2B-GFP, pBabe-Puro, PSM-RB, p27kip1, and p16ink4a, have been previously described (22, 25, 35).

Cell Fusions. Approximately 3×10^5 drug-resistant C33A and SAOS-2 cells were combined in a 50-ml conical tube, centrifuged at $1,000 \times g$, resuspended in 5 ml DMEM, and then centrifuged at $1,000 \times g$. The cells were resuspended in 1 ml of fusion buffer (50% polyethylene glycol 1450 and 50% DMEM) and incubated for 1 min at room temperature. The cells were then washed in 10 ml of DMEM and plated. After fusion, both puromycin and G418 were added to the growth media.

Immunoblotting. Approximately 1×10^6 cells were plated in 10-cm dishes 24 h before transfection. Cells were cotransfected with effectors and either a puromycin selectable plasmid or the H2B-GFP expression plasmid. Transfected cells were subsequently selected with puromycin or sorted by using a fluorescence-activated cell sorter. Immunoblotting was carried out by using standard procedures with the following antibodies: against Cdk2, cyclin A, cyclin E, Cdk4 (Santa Cruz Biotechnology) anti-Flag (Zymed), anti-p53 (K. Fukasawa, University of Cincinnati, Cincinnati, OH), anti-vimentin (W. Ip, University of Cincinnati), and anti-BRG-1 (W. Wang, National Institutes of Health, Bethesda, MD).

Reporter Assays, BrdUrd Incorporation, and Flow Cytometry. Reporter assays, BrdUrd incorporation, and flow cytometry were performed as previously described (14, 23).

Results

Generation and Characterization of Somatic Cell Fusions. It has been hypothesized that all cells, including tumor cells, must inactivate RB to traverse the G_1/S transition (1–4). We have previously reported that whereas the vast majority of tumor cell lines are arrested by introduction of constitutively active RB (PSM-RB), C33A (HPV-negative cervical carcinoma) cells are resistant to PSM-RB-mediated arrest (25). Presumably, a protein that inactivates RB could be responsible for the resistance of C33A cells. Alternatively, C33A could fail to undergo RB-dependent arrest because of the loss of a critical RB cofactor. To delineate between these possibilities, somatic cell fusion experiments were performed. As summarized in Fig. 1*A*, C33A cells were fused to SAOS-2 (osteosarcoma) cells, which we have previously demonstrated to be sensitive to PSM-RB-mediated cell cycle inhibition (25). Initially, stable neomycin and puromycin drug-resistant populations of both C33A and SAOS-2 cells were generated and pooled. Resulting drug-resistant cells were then fused by using polyethylene glycol to generate "homofusions" of C33A cells (C-C) or SAOS-2 cells (S-S) and "heterofusions" of C33A with SAOS-2 (C-S) (Fig. 1*A*). The fusion populations were propagated in the presence of dual (puromycin-G418) selection for 2 weeks, at which point all unfused cells where killed by either G418 or puromycin (not shown). Giemsa staining of the fused cells revealed that the homofusions were slightly larger than parental cells but maintained similar morphologies (Fig. 1*B*).

However, the heterofusion cells resulted in a large cell with SAOS-2-like morphology (Fig. 1*B*). To ensure that the hybrid cells represent faithful fusion of their parental counterparts, biochemical analyses were performed. As shown in Fig. 1*C*, immunoblot analysis revealed that the mutated form of the nuclear p53 tumor suppressor gene (expressed only in C33A cells, lane 1) and the cytoskeletal protein vimentin (found only in SAOS-2 cells, lane 2) were both expressed in the C-S populations (lanes 3–6) (36, 37). Immunohistochemistry also revealed that the homofusions of the parental cells (C-C, S-S) were not affected in their ability to express p53 (99%) and vimentin (100%), respectfully (Fig. 1*D*). Virtually 100% of C-S fusions expressed both p53 and vimentin, indicating successful fusion of C33A to SAOS-2. Lastly, the fused cells were analyzed by flow cytometry, which revealed that both the homofusions as well as the heterofusions maintained a G_1 DNA content of 4N, whereas the parental cells demonstrated 2N DNA content (Fig. 1*E*). Four independent C-S fusion populations were generated, and C-S fusions nos. 1–4 all behaved identically (data not shown). Thus, the data for C-S fusion no. 1 is representative of the polyclonal fusion populations. Together, these data affirm the generation of valid C33A-SAOS-2 heterofusions.

Restoration of RB Signaling to Cyclin A Is Stimulated by Dominant Genetic Factors. The repression of E2F-mediated transcription is thought to underlie RB-mediated cell cycle arrest (1). We therefore examined the effect of PSM-RB on E2F activity in the fused cells. Interestingly, ectopic expression of PSM-RB in the C-C, S-S, and C-S fusions resulted in the inhibition of E2F reporter activity by 71.5%, 92.8%, and 71%, respectively, compared with cells transfected with empty vector (Fig. 2*A*). These data demonstrate that RB retains the ability to inhibit E2F activity in C33A cells, but this inhibition is not sufficient to induce cell cycle arrest (38). Previous reports have shown that the ability of RB to signal to attenuate cyclin A/Cdk2 activity is essential for arresting cells at the G_1/S transition (14, 23, 38). We therefore investigated the effect of PSM-RB on the cyclin A promoter (Fig. 2*B*). For these experiments, PSM-RB expression plasmid or parental vector was cotransfected with a human cyclin A reporter (−608cycA/Luc) plasmid into C-C, S-S, and C-S cells. Expression of PSM-RB in the C-C hybrid did not affect cyclin A-dependent transcriptional activity (*Left*), whereas in the S-S line, expression of PSM-RB inhibited cyclin A reporter activity by 94.6% when compared with vector (*Center*). In the C-S hybrid lines, expression of PSM-RB inhibited cyclin A transcriptional activity by 86.2% when compared with vector (*Right*). These data demonstrate that SAOS-2 cells contain a trans-acting factor capable of restoring RB-mediated signaling to the cyclin A promoter in C33A cells.

To confirm these observations, the effect of RB on endogenous G_1 Cdk/cyclin expression was determined. The C-C, S-S, and C-S cell lines were cotransfected with parental vector or PSM-RB expression plasmid and a histone-H2B-GFP expression plasmid. Transfected cells were specifically isolated by fluorescence-activated cell sorting, and visual immunofluorescence microscopy revealed >90% GFP-positive cells after sorting (data not shown). Collected cells were lysed and subjected to immunoblot analyses (Fig. 2*C*). Expression of PSM-RB in the C-C cell line had no effect on the levels of endogenous cyclin A or Cdk2 protein compared with cells transfected with parental vector (compare lanes 1 and 2). In S-S cells, ectopic expression of PSM-RB caused a decrease in both endogenous cyclin A and Cdk2 protein (compare lanes 3 and 4). Consistent with data from the reporter assays (Fig. 2*B*), transfection of PSM-RB caused an inhibition in cyclin A protein levels in the C-S heterofusions (compare lanes 5 and 6). PSM-RB also caused a modest reduction in the levels of Cdk2 protein (compare lanes 3 and 4 and lanes 5 and 6). In all cells tested, no changes were observed on

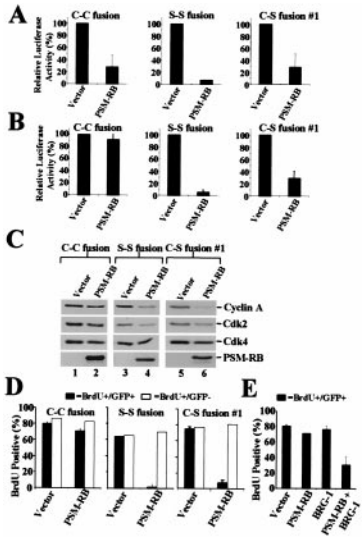

Fig. 2. A dominant genetic determinant is important for RB signaling. (*A*) The C-C, S-S, and C-S cell fusions were transfected with the E2F promoter reporter plasmid E2F/Luc, CMV-β-galactosidase (β-gal), and vector or PSM-RB. (*B*) The C-C, S-S, and C-S cell fusions were transfected with the cyclin A promoter reporter plasmid −608Luc, CMV-β-gal, and either vector or PSM-RB. (*C*) Fusion cells were cotransfected with H2B-GFP and either CMVNeoBam (lanes 1, 3, and 5) or PSM-RB (lanes 2, 4, and 6). Forty-eight hours after transfection, the transfected cells were sorted from the untransfected cells by fluorescence-activated cell sorting for GFP fluorescence. Lysates were prepared from these cells, and protein was resolved by SDS/PAGE and then immunoblotted for cyclin A, Cdk2, cyclin E, and Cdk4 proteins. (*D*) The C-C, S-S, and C-S cell fusions were cotransfected with a H2B-GFP expression plasmid and either vector or PSM-RB. BrdUrd was added 48 h after transfection and cells stained for BrdUrd incorporation. The displayed values were determined from two independent experiments with at least 150 transfected (GFP positive; solid bars) or 150 untransfected (GFP negative; open bars) cells counted per experiment. (*E*) The C-C cell fusions were cotransfected with H2B-GFP expression plasmid and either BRG-1 or PSM-RB + BRG-1.

PSM-RB transfection in the levels of cyclin E (data not shown) or Cdk4 (lanes 1–6). Collectively, these data demonstrate that RB signaling to Cdk2/cyclin A is disrupted in C33A cells because of the lack of a specific trans-acting factor, which can be supplied by SAOS-2 cells to restore this signaling pathway.

Because cyclin A is a critical target in RB-mediated arrest, restoration of RB signaling to cyclin A should restore RB-mediated cell cycle inhibition in C33A cells. To examine this, C-C, S-S, and C-S cells were cotransfected with plasmids encoding H2B-GFP and either empty vector or PSM-RB and scored for the ability of transfected (GFP-positive) cells to incorporate BrdUrd (Fig. 2*D*). C-C cells transfected with PSM-RB were not inhibited in their ability to enter DNA synthesis as compared with untransfected (GFP-negative) cells from the same coverslip or cells transfected with vector alone (*Left*). By contrast, BrdUrd incorporation was abolished in S-S cells transfected with PSM-RB (*Center*). The C-S fusion cells transfected with PSM-RB also failed to incorporate BrdUrd (*Right*), consistent with the hypothesis that SAOS-2 cells provide a dominant trans-acting factor that restores RB activity.

It has been previously shown that ectopic overexpression of BRG-1, a SWI/SNF family member, can enhance RB function (30–32, 38, 39). C33A cells are reported to be deficient in BRG-1 (30). Therefore, we attempted to restore RB activity in C33A cells by coexpression of PSM-RB with BRG-1 (Fig. 2*E*). C-C cells were cotransfected with H2B-GFP and BRG-1, PSM-RB, or empty vector. All transfected cells incorporated BrdUrd at similar rates. However, coexpression of both PSM-RB and BRG-1 in the C-C fusion resulted in a 62% inhibition of BrdUrd

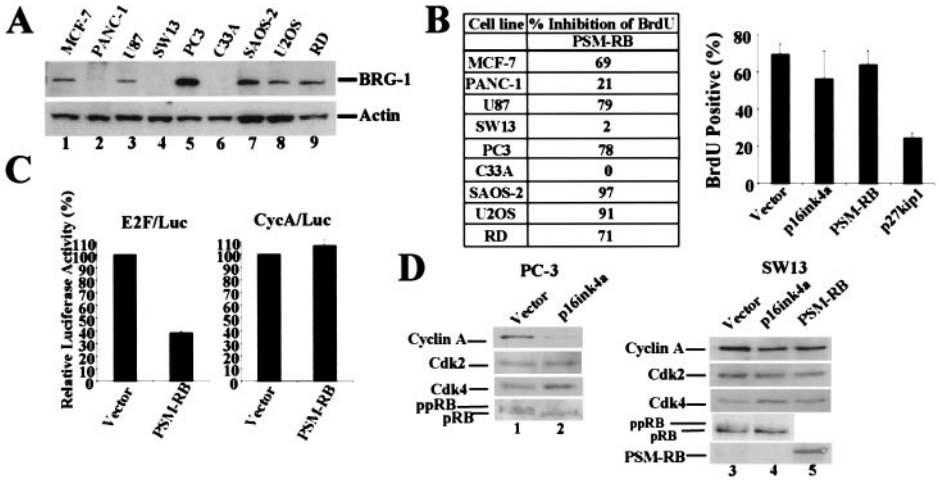

Fig. 3. BRG-1 deficiency renders tumor cell lines resistant to RB-mediated signaling and arrest. (*A*) Thirty micrograms of total protein from MCF-7, PANC-1, U87, SW13, PC3, C33A, SAOS-2, U2OS, and RD was separated by 7.5% SDS/PAGE and immunoblotted for BRG-1 and actin. (*B*) MCF-7, PANC-1, SW13, U87, PC3, C33A, SAOS-2, U2OS, and RD were cotransfected with GFP expression plasmid and either vector or PSM-RB. Cells were labeled with BrdUrd, and the percentage of inhibition of BrdUrd incorporation by PSM-RB relative to vector is reported. The data for MCF7, U87, PC3, RD, and U2OS have been previously published (25) and are shown for illustrative purposes. (*B*) SW13 cells were cotransfected with H2B-GFP expression plasmid and plasmids encoding vector, PSM-RB, p16ink4a, or p27Kip1. Cells were labeled with BrdUrd, and the percentage of transfected cells incorporating BrdUrd is shown. (*C*) SW13 cells were transfected with E2F/Luc, CMV-β-gal, and PSM-RB plasmids. SW13 cells were also transfected with the cyclin A promoter reporter plasmid −608Luc, CMV-β-gal, and PSM-RB. (*D*) SW13 and PC3 cells were cotransfected with either vector, p16ink4a, or PSM-RB along with pBabe-PURO. Twenty-four hours after transfection, cells were subjected to puromycin selection. Selected cells were harvested, and equal total protein was resolved by SDS/PAGE and then subjected to immunoblot analysis for RB, cyclin A, Cdk2, cyclin E, and Cdk4 protein.

incorporation. These data demonstrate that like fusion with SAOS-2, BRG-1 can restore RB activity in C33A cells. Because SAOS-2 cells express functional BRG-1, we hypothesized that BRG-1 is the dominant factor present in SAOS-2, which restores RB activity in C-S cells. This hypothesis predicts that BRG-1 loss disrupts the antiproliferative action of RB.

BRG-1 Mediates RB Signaling to Cyclin A to Induce Cell Cycle Arrest. To determine whether loss of BRG-1 in tumor cells disrupts the ability of RB to inhibit cell cycle progression, a panel of tumor cell lines were screened for their relative expression of BRG-1. Consistent with previous reports, BRG-1 protein expression is dramatically reduced/absent in C33A and SW13 cells (Fig. 3*A*) (30). The PANC-1 cell line expressed considerably reduced levels of BRG-1, relative to the majority of tested cell lines. Greater than 20 additional cell lines were clearly positive for BRG-1, for six of which data are shown (MCF-7, U87, PC3, SAOS-2, U2OS, and RD). These cells have been previously tested for their ability to incorporate BrdUrd after transfection with either empty vector or PSM-RB (25). All BRG-1-positive cells tested were significantly inhibited (69–97%) for BrdUrd incorporation after transfection with PSM-RB (Fig. 3*B Left*). Strikingly, however, BRG-1-deficient cells (C33A, SW13, and PANC-1) were not efficiently inhibited for BrdUrd incorporation (0–21%) after transfection with PSM-RB (Fig. 3*B Left*).

As described above, the downstream target of RB-mediated arrest is Cdk2/cyclin A. To determine whether BRG-1-defective cells still require Cdk2 function for cell cycle progression, SW13 cells were transfected with plasmids encoding H2B-GFP and either p16ink4a, PSM-RB, or p27Kip1, and scored for the ability to incorporate BrdUrd (Fig. 3*B Center* and *Right*). SW13 cells continued to incorporate BrdUrd after transfection with either vector, p16ink4a (which activates the endogenous RB), or PSM-RB. By contrast, cells transfected with p27Kip1, which is a direct inhibitor of Cdk2, were inhibited for BrdUrd incorporation, and similar results have been observed in C33A cells (38, 39). Therefore, the requirement for Cdk2 is maintained in BRG-1-defective cells, suggesting that the resistance to RB-mediated arrest of these cells is because of a specific defect in RB signaling to attenuate Cdk2.

Because overexpression of active RB was ineffective at inhibiting cell cycle progression in BRG-1-defective cells (Fig. 3*B*), we assessed the ability of RB to modulate E2F and cyclin A promoter activity in SW13 cells (Fig. 3*C*). To do so, SW13 cells were cotransfected with either empty vector or PSM-RB and the E2F/Luc reporter plasmid. These experiments revealed that active RB inhibited E2F transcriptional activity by 62% when compared with vector (*Left*). These data demonstrate that inhibition of E2F is insufficient to cause RB-mediated arrest and are consistent with our previous observations (Fig. 2*D*). Because we have observed that cyclin A and Cdk2 expression is attenuated in cells arrested by RB, we determined whether RB could signal to cyclin A in SW13 cells. Cotransfection of either empty vector or PSM-RB with −608CycA/Luc into SW13 cells revealed that RB signaling to cyclin A was abrogated, as evidenced by the inability of active RB to attenuate cyclin A promoter activity. To verify this effect at the level of protein, SW13 and PC-3 cells (as a positive control for RB arrest) were cotransfected with empty vector, p16ink4a, or PSM-RB and a vector carrying puromycin resistance. Transfected cells were rapidly selected with puromycin, harvested, and immunoblotted for endogenous cell cycle components. As expected, PC-3 cells transfected with p16inka demonstrate underphosphorylated RB and reduced cyclin A and Cdk2 proteins levels, whereas levels of Cdk4 remained unchanged (Fig. 3*D Left*). Although the endogenous RB was underphosphorylated in p16ink4a-transfected SW13 cells, no alteration in cyclin A or Cdk2 protein levels was observed (Fig. 3*D Right*). These data demonstrate that RB does not signal to Cdk2/cyclin A in BRG-1-deficient SW13 cells.

In an attempt to restore RB signaling to cyclin A, expression plasmids encoding BRG-1 and PSM-RB were cotransfected with −608CycA/Luc into SW13 cells, and the ability of PSM-RB to signal to the cyclin A promoter was assessed by reporter assay (Fig. 4*A*). Although PSM-RB alone did not affect cyclin A promoter activity, coexpression of BRG-1 and PSM-RB resulted in an 83% decrease in cyclin A transcriptional activity when

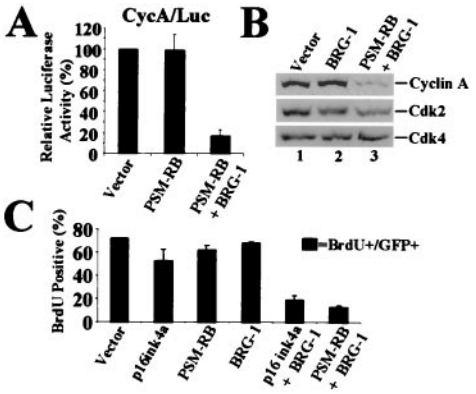

Fig. 4. BRG-1 expression restores RB-mediated arrest in BRG-1 deficient cells. (*A*) SW13 cells were cotransfected with the −608Luc, CMV-β-gal, and vector, PSM-RB or PSM-RB + BRG-1. Samples were then processed for luciferase activity and normalized to β-gal activity. (*B*) SW13 cells were cotransfected with either vector, BRG-1, or PSM-RB + BRG-1 along with pBabe-PURO. Transfected cells were selected with puromycin, and protein lysates were resolved by SDS/PAGE and subjected to immunoblotting for cyclin A, Cdk2 and Cdk4. (*C*) SW13 cells were cotransfected with either vector, p16ink4a, PSM-RB, BRG-1, p16ink4a + BRG-1, or PSM-RB + BRG-1 and H2B-GFP expression plasmids. Cells were labeled with BrdUrd, and the percent of transfected cells incorporating BrdUrd is shown.

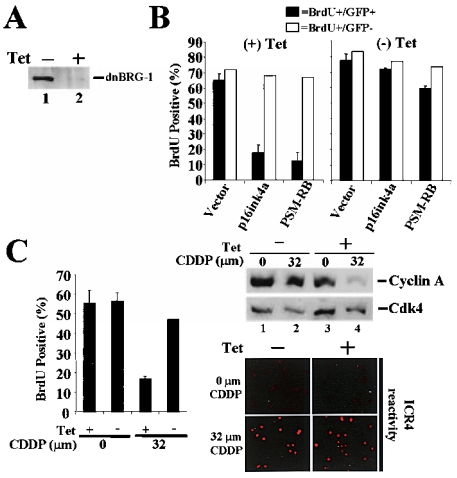

Fig. 5. Expression of dominant-negative BRG-1 prevents RB-mediated arrest. (*A*) B05-1 cells were cultured in either the absence or presence of 2 μg/ml tetracycline. Cells were harvested, equal total protein was resolved by SDS/PAGE, and dnBRG-1 protein was detected by anti-FLAG immunoblotting. (*B*) B05–1 cells cultured in the absence or presence of tetracycline were cotransfected with either vector, PSM-RB, or p16ink4a expression plasmids and the H2B-GFP expression plasmid. Cells were labeled with BrdUrd and stained for the incorporation of BrdUrd. (*C*) B05–1 cells cultured in either the absence or presence of tetracycline were treated with 0 or 32 μM CDDP for 16 h. (*Left*) Cells were then labeled with BrdUrd for 5 h, at which point they were fixed and stained for BrdUrd incorporation. (*Center*) Cells were fixed and stained for platinated DNA with the ICR4 antibody. Shown are photomicrographs taken at equivalent exposures. (*Right*) Cells were lysed, and equal total protein was resolved by SDS/PAGE. Cyclin A and Cdk4 protein levels were determined by immunoblotting.

compared with vector-transfected cells. Next, we verified this finding by examining the protein expression of endogenous cell cycle targets. To do this, SW13 cells were transfected with either vector, BRG-1, or PSM-RB + BRG-1 along with a puromycin resistance plasmid (Fig. 4*B*). After rapid selection, the transfected cells were harvested and subjected to immunoblot analysis. Ectopic expression of BRG-1 alone had little effect on endogenous cyclin A, Cdk2, or Cdk4, whereas expression of PSM-RB + BRG-1 was sufficient to attenuate cyclin A/Cdk2 protein levels without affecting Cdk4 or cyclin E (data not shown) (Fig. 4*B*). These data indicate that coexpression of BRG-1 restores RB signaling to cyclin A in SW13 cells. To determine whether BRG-1 restores cell cycle arrest, SW13 cells were cotransfected with plasmids encoding H2B-GFP and either PSM-RB, BRG-1, or both, and were scored for the ability of the transfected cells to incorporate BrdUrd. Cells transfected either with vector, BRG-1, p16ink4a, or PSM-RB alone incorporated BrdUrd at approximately equal rates (Fig. 4*C*). However, cotransfection of BRG-1 and either p16ink4a or PSM-RB resulted in a significant decrease in cell cycle progression. These findings indicate that the ability of BRG-1 to mediate RB signaling to cyclin A/Cdk2 is required for the antiproliferative action of RB.

Expression of Dominant-Negative BRG-1 Reverses RB-Mediated Cell Cycle Arrest. The data presented correlate BRG-1 expression with the ability of RB to signal to cyclin A and cause cell cycle arrest. To determine directly whether the antiproliferative action of RB requires BRG-1, NIH 3T3 cells, which exhibit tetracycline-regulated expression of dominant-negative BRG-1 (B05–1), were used (33). On removal of tetracycline from the culture media, the expression of dnBRG-1 was induced (Fig. 5*A*). The B05–1 cells cultured in either the presence or absence of tetracycline were cotransfected with plasmids encoding H2B-GFP and either p16ink4a or PSM-RB and were monitored for the ability to incorporate BrdUrd (Fig. 5*B*). In the presence of tetracycline, ectopic expression of p16ink4a and PSM-RB inhibited BrdUrd incorporation by 73% and 81%, respectively (Fig. 5*B Left*). By contrast, ectopic expression of p16ink4a and PSM-RB did not significantly inhibit BrdUrd incorporation in the absence of tetracycline (presence of dnBRG-1) (Fig. 5*B Right*). Therefore, these data demonstrate that active BRG-1 is required for RB-mediated cell cycle arrest.

Ectopic expression of p16ink4a and PSM-RB is known to mimic the physiological activation of endogenous RB (3, 21). To determine whether BRG-1 is required for RB-dependent cell cycle inhibition mediated by a physiological stress, we explored the response of the B05–1 cells to the DNA-damaging agent cisplatin (CDDP). It has previously been shown that RB is required for the cell cycle inhibitory response to CDDP, and that the activation of RB by CDDP leads to an RB-dependent reduction in cyclin A expression (40, 41). In the presence of tetracycline, the expression of dnBRG-1 is repressed, and treatment with 32 μM CDDP resulted in the inhibition of BrdUrd incorporation (Fig. 5*C Left*). In contrast, in the absence of tetracycline, where dnBRG-1 expression was induced, treatment with 32 μM CDDP had minimal effect on BrdUrd incorporation. This result was virtually identical to what is observed in Rb−/− cells, which fail to respond to this dose of CDDP (not shown and ref. 40). Analysis of the degree of platinated DNA was carried out with the ICR4 antibody (42), which recognizes platinum-DNA adducts (Fig. 5*C Center*) and no significant difference detected. Thus the observed failure to arrest was not because of differential repair of the CDDP damage. Lastly, to address the mechanism through which dnBRG-1 bypasses CDDP-mediated cell cycle inhibition, cyclin A and Cdk4 protein levels were monitored (Fig. 5*C Right*). These results showed that CDDP leads to the down-regulation of cyclin A expression only in the absence of dnBRG-1. Collectively, these data indicate that RB-dependent signaling and cell cycle inhibition achieved through a physiological stress also depend on BRG-1.

Discussion

In cancer cells, it is known that RB is inactivated via deregulation of upstream signaling that occurs through either loss of p16ink4a

or overproduction of Cdk4 or cyclin D1 (1–4). The overproduction of Cdk4/cyclin D (as occurs through gene amplification and other mechanisms) or loss of p16ink4a each result in deregulated RB phosphorylation. This leads to the disruption of RB protein binding and transcriptional repression activity (1–4). Here we show that BRG-1 deficiency bypasses cell cycle inhibition elicited by activation of the RB pathway. PSM-RB and p16ink4a fail to initiate cell cycle arrest in BRG-1-deficient cells (SW13, C33A, and PANC-1) or in cells harboring a dominant-negative mutant of BRG-1. Similarly, disruption of BRG-1 abrogated the RB-dependent cell cycle inhibition after CDDP treatment. Together, these results indicate that disruption of the RB pathway also occurs downstream of RB through the loss of a critical factor required for RB-mediated transcriptional repression.

The mechanism underlying the loss of BRG-1 protein in tumor cells is not understood. The gene for BRG-1 has not been found to be deleted in C33A, PANC-1, or SW13 cells, and it is possible to detect a low level of BRG-1 protein in PANC-1 and C33A cells (30, 31, 43). This suggests that mechanisms other than genetic loss are responsible for the attenuated expression of BRG-1 in these cell lines. All studies carried out to date indicate that BRG-1 is required for cellular viability (44). This suggests that genetic loss of BRG-1 would be selected against, whereas alternative mechanisms to lower the amount of BRG-1 and thus disrupt its ability to function in the RB-pathway would be selected for.

Transcriptional repression by RB involves transcription factors such as E2F (1–4). The recruitment of histone deacetylases is believed to be the mechanism through which RB leads to transcriptional repression (12, 13). On the basis of our observations, we conclude that although this simple description is sufficient to describe RB-mediated repression of E2F/Luc, it may not accurately describe how RB mediates the repression of the cyclin A promoter. Although BRG-1 is known to enhance RB function as an inhibitor of E2F-mediated transcription (31), our data indicate that BRG-1 is required for RB to inhibit both endogenous cyclin A protein expression and cyclin A promoter activity. Currently, it is unclear how BRG-1 functions to assist RB-mediated repression. BRG-1 is a member of the mammalian SWI/SNF complex, which has ATP-dependent chromatin remodeling capabilities (28, 29). The dnBRG-1 allele used in this study is specifically defective in ATPase activity, suggesting that the inactivation of the SWI/SNF complex renders cells resistant to RB-mediated growth inhibition. Therefore, loss of other members of this complex such as SNF5/Baf47/INI1, which is lost in rhabdoid tumors, may also disrupt RB function (43, 45).

Disruption of the RB growth inhibitory pathway has been postulated to occur in virtually all tumors (3, 14). Although cyclin D, Cdk4, p16ink4a, and RB are lost in a large fraction of human tumors, additional lesions that disrupt RB signaling are likely involved in those tumors that do not exhibit mutation/deregulation of any of these proteins. This study shows that loss of BRG-1 disrupts the ability of RB to signal and is therefore analogous to RB loss. As such, we demonstrate a mechanism through which RB is disrupted in cancer via the abolition of RB-dependent signaling.

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