BRCA1-associated growth arrest is RB-dependent

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***BRCA1* is a susceptibility gene for breast and ovarian cancer with growth-inhibitory activity for which the mechanism of action remains unclear. When introduced into cells, *BRCA1* inhibits growth of some but not all cell lines. In an attempt to uncover the mechanism of growth suppression by *BRCA1*, we examined a panel of cell lines for their ability to reduce colony outgrowth in response to *BRCA1* overexpression. Of all variables tested, only those cells with wild-type pRb were sensitive to *BRCA1*-induced growth suppression. In cells with an intact *rb* gene, inactivation of pRb by HPV E7 abrogates the growth arrest imposed by *BRCA1*. In accordance with these observations, we found that *BRCA1* could not suppress BrdUrd uptake in primary fibroblasts from *rb*−/− mice and exhibited an intermediate ability to inhibit DNA synthesis in *rb*+/− as compared with *rb*+/+ cells. We further found that the BRCA1 protein complexes with the hypophosphorylated form of pRb. This binding is localized to amino acids 304–394 of BRCA1 protein and requires the ABC domain of pRb. In-frame deletion of BRCA1 fragment involved in interaction with pRb completely abolished the growth-suppressive property of *BRCA1*. Although it has been reported that *BRCA1* interacts with p53, we find the p53 status did not affect the ability of *BRCA1* to suppress colony formation. Our data suggest that the growth suppressor function of *BRCA1* depends, at least in part, on Rb.**

Germline mutations in *BRCA1* are found in ≈50–60% of hereditary breast and ovarian cancers (1). Despite the unequivocal role of *BRCA1* in familial breast cancer susceptibility, the biological function of the BRCA1 protein remains unclear. Experimental data suggest that BRCA1 may be a negative regulator of cell growth. Attenuation of BRCA1 synthesis by antisense oligonucleotide increased the proliferative rate of both benign and malignant mammary epithelial cells in culture (2), and *BRCA1* expression decreased the capacity of MCF7 breast cancer cells to form tumors in nude mice (3). However, *BRCA1* expression increases as cells progress through the G_1 and S phases of the cell cycle (4, 5). Furthermore, homozygous BRCA1 mutant mice died at day 7.5 of embryogenesis with evidence of abnormal cessation of cellular proliferation accompanied by high levels of the CDK inhibitor p21 and low levels of cyclin E and mdm2 (6, 7). Recently, colocalization and physical interaction of BRCA1 with Rad51 protein has raised the possibility that BRCA1 is involved in DNA repair (8). Moreover, the COOH terminus of the BRCA1 protein can activate transcription in *in vitro* experiments (9, 10) and coactivate transcription of p53-regulated genes (11, 12).

The experimental biology of BRCA1 therefore suggests that BRCA1 may have different functions, each best manifested in specific study systems and cell lines. Herein, we show that BRCA1 binds preferentially to the hypophosphorylated form of Rb and that the growth-suppressive phenotype of BRCA1 depends on the presence of a functional Rb protein. These data indicate the complexity of BRCA1 action given its reliance on other molecules to induce a biological response.

Methods

Cells and Cell Culture. Mcf7 (breast carcinoma) and HBL100 (normal breast epithelial cells immortalized with SV40) were obtained from the Tissue Culture Facility, Lineberger Cancer Center, University of North Carolina at Chapel Hill. U2OS, SaOS2 (osteosarcoma), and HaCaT (immortalized human keratinocytes) were from Y. Xiong (University of North Carolina at Chapel Hill). UNC7 and JHU012 (head and neck cancer) were a gift from W. Yarbrough (University of North Carolina at Chapel Hill). H2009 (lung cancer) was obtained from F. Kaye (National Cancer Institute, Bethesda). Mouse embryo fibroblasts derived from Rb+/− and Rb−/− as well as wild-type control were from Tyler Jacks (Massachusetts Institute of Technology, Boston).

Plasmid Constructs. Amino acids 303–394 were deleted from BRCA1 protein with the GeneEditor *in vitro* site-directed mutagenesis system (Promega) with the use of oligonucleotide 5′-CCCATCATGTGAGTCATCAGAAGCCTTTTCTACATTCATTCT-3′ according to manufacturer's instructions. COOH-terminal truncation of BRCA1 was made by deletion from internal *Apa*I to the *Apa*I site in the polylinker region of pcDNA3 vector.

Glutathione *S*-Transferase (GST) Protein Purification and GST Pull-Down Assay. Glutathione–Sepharose 4B was purchased from Pharmacia, and recombinant GST fusion proteins were purified according to the manufacturer's instructions. Purified GST fusion proteins attached to beads were equalized by protein amount with Coomassie-stained gel, and the total amount of beads was balanced with naked glutathione–Sepharose beads. Approximately 2 μg of purified GST fusion proteins was used per reaction.

For experiments shown in Fig. 6, ≈1 μg of corresponding plasmid was *in vitro* translated in presence of [^{35}S]methionine with a TNT kit (Promega) according to the manufacturer's instruction. For the control of protein synthesis, 2 μl of each reaction was loaded in lanes labeled IVT. Ten microliters of the reaction mixture was diluted in binding buffer (50 mM Tris·HCl, pH 7.5/150 mM NaCl/0.5% Nonidet P-40/50 mM NaF/25 mg/ml aprotinin/25 mg/ml leupeptin/1 mM benzamidine/1 mM DTT), combined with GST fusion protein-coated beads, and rotated for 1 h at 4°C. Beads were washed five times with binding buffer, boiled with 50 μl of loading dye, and separated by PAGE (10% gel). Proteins were visualized with autoradiography.

For protein pull-down assay presented in Fig. 7, ≈2×10^7 U2OS or SK-N-SH cells were used per reaction. Cells were lysed in HNTG buffer (20 mM Tris·HCl, pH 8.0/150 mM NaCl/1.5 mM $MgCl_2$/0.2 mM EDTA/10% glycerol/0.5% NP-40/50 mM NaF/protease inhibitors as above) for 30 min, centrifuged for 10 min at 11,000 × *g*, and incubated with beads coated with different GST fusion proteins balanced by the amount of protein as described above. After 2 h, the beads were washed five times with HNTG buffer, boiled with 50 μl of Laemmli sample buffer, and

Abbreviations: GST, glutathione *S*-transferase; β-gal, β-galactosidase.

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loaded onto 6% PAGE gels. Proteins were visualized by Western blotting with anti-BRCA1 antibody C-20 (Santa Cruz) or anti-Rb G3-245 (PharMingen).

To prove that BRCA1 specifically binds to GST-Rb ABC protein, the beads after pull-down reaction were treated with 20 mM glutathione for 10 min at room temperature to disrupt the interaction of GST with the beads, washed once, and boiled with 50 μl of loading dye as above.

Immunoprecipitation and Western Blotting. To coimmunoprecipitate the endogenous BRCA1-pRb complex, $\approx 4 \times 10^7$ U2OS cells were used for one reaction. Cells were lysed with modified HNTG buffer that contained 1.5 mM $ZnCl_2$ but no NP-40 and disrupted by sonication in an Ultrasonic processor (Misonix, Farmingdale, NY). BRCA1 was immunoprecipitated with 4 μg of anti-BRCA1 antibody (MS110, Calbiochem) in the presence of protein A/G-Sepharose (Santa Cruz) overnight. Rb was immunoprecipitated with either 40 μl of agarose-conjugated C-36 antibody (Calbiochem) or 4 μg of anti-Rb C-15 antibody (Santa Cruz) with protein A–Sepharose (Pierce). Immune complexes were washed five times with lysis buffer plus 0.5% NP-40 and resolved on a 6% gel for BRCA1.

Transfections and Immunohistochemical Staining. For transfection, cells were split the day before in 60-mm plates and transfected at $\approx$60% confluency with Lipofectamine reagent (GIBCO/BRL) according to manufacturer's instructions. To achieve an equimolar amount of transfected DNA, 5 μg of pcDNA3-BRCA1 or 2.5 μg of pcDNA3 (Invitrogen) was used. The total amount of DNA was balanced with carrier DNA. In 2 days, cells were split at different dilutions into selection media containing G-418. From 2 to 3 weeks later, colonies were stained with crystal violet and counted.

For transfection of the U2OS or HBL100 cell lines with linearized plasmids, 20 μg of pcDNA3-BRCA1 vector was digested with either *Pvu*I or *Hin*dIII–*Afl*II restriction enzymes. Completeness of digestion was verified by gel electrophoresis. DNA was ethanol-precipitated and dissolved in 10 mM Tris/1 mM EDTA buffer, pH 8.0, and the concentration was determined. Five micrograms of each sample was transfected by using Lipofectamine reagent (GIBCO/BRL).

U2OS cells expressing HPV16-E7 were obtained by transfection with pCEP4-E7 plasmid (Y. Xiong, University of North Carolina at Chapel Hill), cells were selected with 200 μg/ml hygromycin, and resistant clones were pooled and maintained in selection media. Expression of E7 protein was verified by reverse transcription–PCR.

U2OS cells overexpressing p53Val135 mutant were produced by cotransfection with LTRp53cG(val) (13) and pLXSP carrying the puromycin resistance gene. Clones were selected in 1 μg/ml puromycin, and the clone expressing the highest level of p53 was selected for further analysis. To test whether p53 function has been compromised in this clone, cells were treated with 0.34 μM Adriamycin (Sigma) for 18–24 h, and p53 and p21 protein induction was analyzed by Western blotting.

Mouse embryo fibroblasts on 60-mm plates were cotransfected with 1 μg of CMV-*lacZ* and 6 μg of BRCA1 expression vector or 3 μg of empty vector by using Lipofectamine Plus reagent (GIBCO/BRL). At 48 h, BrdUrd was added for 12 h. Cells were fixed with 3.7% formaldehyde for 10 min, permeabilized with 0.2% Triton X-100, and stained for β-galactosidase (β-gal) by using a β-gal staining kit (Invitrogen). Histochemical detection of BrdUrd incorporation was done with a cell proliferation kit (Amersham), which uses monoclonal anti-BrdUrd antibody and secondary anti-mouse horseradish peroxidase conjugate.

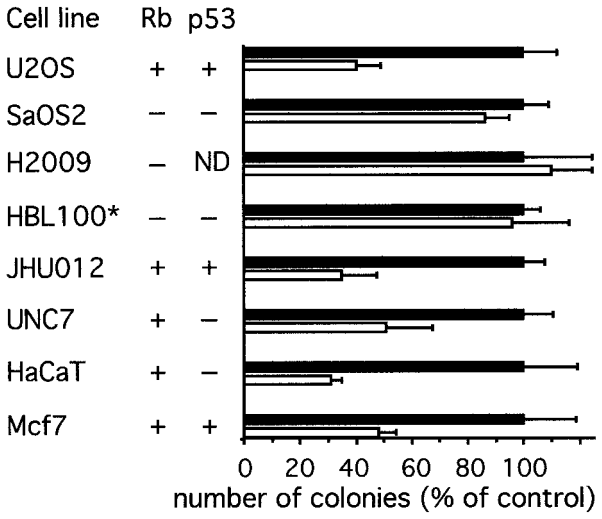

Fig. 1. Growth suppression by *BRCA1*. Cells were transfected with equimolar amounts of pcDNA3-BRCA1 or vector alone and selected with G-418; emerging colonies were stained and counted. The number of colonies obtained with the vector alone was considered as 100%. pRb and p53 status of cells is shown. Each experiment was repeated at least three times, and error bars represent standard deviation. ■, vector alone transfections; □, *BRCA1* transfections. *, HBL100 cell line is SV40-positive; ND, not done.

Adenovirus-Mediated BRCA1 Overexpression. Recombinant adenoviruses were made according the procedure described by He *et al.* (14). U2OS and HBL100 cells were split the day before the infection at 0.6×10^6 cells per 100-mm plate and infected at multiplicity of infection of 500 plaque-forming units/cell for U2OS or 1,000 plaque-forming units/cell for HBL100. Cell cycle distribution was tested 3 days after infection by using FACscan (Becton Dickinson). Nocodazole was added to the cell culture 12 h before fixation at a concentration of 40 ng/ml.

Results

***BRCA1* Suppresses Growth of Rb-Positive Cell Lines.** It has been reported that elevated level of *BRCA1* inhibits growth of breast and ovarian but not lung or colon cancer cell lines (3). We, however, noticed that a number of other cell lines were also growth-arrested when the *BRCA1* gene was overexpressed. To examine what cellular background may affect *BRCA1* function, we transfected *BRCA1* into panel of cell lines with known status of the major growth-regulatory genes, such as p53, pRb. Cells transfected with a BRCA1 expression vector or an equimolar amount of vector alone were selected with G-418 and colonies were stained and counted in 2–3 weeks. Of all variables tested, only Rb status correlated with *BRCA1*-induced growth suppression. Overexpression of *BRCA1* in cells with wild-type pRb resulted in a 50–65% reduction in the number of colonies compared with vector alone (Fig. 1). Three cell lines with either a deletion in the *Rb* gene (SaOS2 and H2009) or inactivation of pRb because of the presence of SV40 (HBL100) showed no difference in the number of colonies after transfection with *BRCA1* compared with the control.

The colony formation assay used in our studies is based on the comparison of the number of colonies originated by cells transfected with the BRCA1 expression vector (pcDNA3-BRCA1) and an equimolar amount of empty vector (pcDNA3). This is accurate only if we assume that plasmids of different molecular weight (pcDNA3 and pcDNA3-BRCA1) will be transfected into the cell with the same efficiency. To validate the method, we performed colony formation assays by using pcDNA3-BRCA1 to achieve both the *BRCA1* baseline and the *BRCA1*-

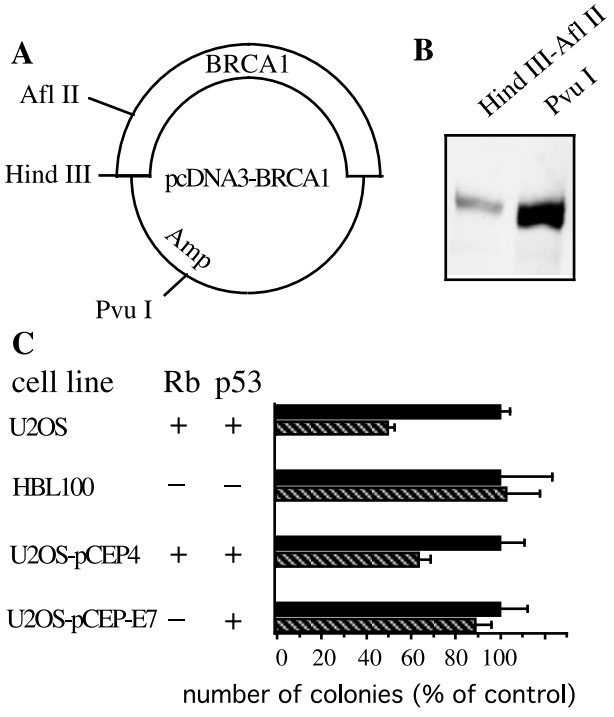

Fig. 2. Colony formation assay was performed with the pcDNA3-BRCA1 plasmid linearized at different sites for positive and negative control. (*A*) Restriction map of the plasmid used in this experiment. (*B*) Western blot analysis of *BRCA1* expression from differentially cleaved pcDNA3-BRCA1 in Cos-7 cells. (*C*) Colony formation assay with linearized plasmids. ■, no *BRCA1* control (*Hin*dIII–*Afl*II digest). ▧, overexpression of *BRCA1* (*Pvu*I digest).

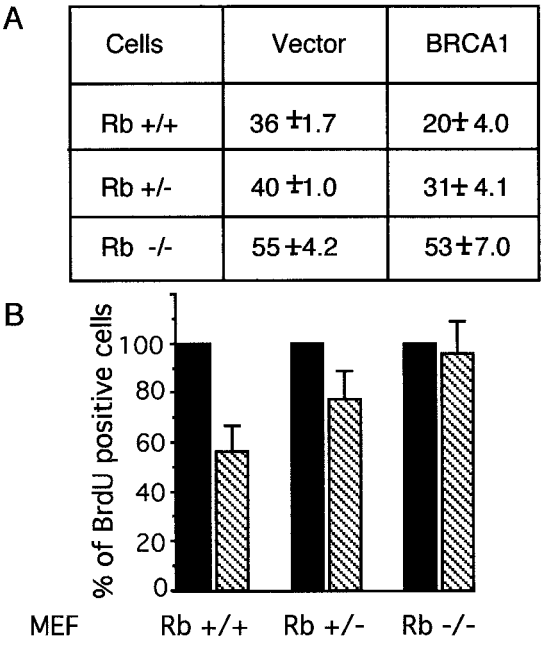

Fig. 3. Proliferation rate of primary mouse embyo fibroblasts transfected with *BRCA1*. Fibroblasts derived from *rb*+/+, *rb*−/−, or *rb*+/− were cotransfected with a *lacZ* expression vector and BRCA1 or vector alone. In 2 days, cells were labeled with BrdUrd and stained for β-gal and BrdUrd. Cells positive for β-gal were scored for proliferation assessed by BrdUrd incorporation. (*A*) Percentage of proliferating cells in the population of transfected cells. (*B*) Same as in *A* with the number of proliferating cells in each cell line transfected with *lacZ* and vector alone taken as a 100%. ■, transfection with vector alone; ▧, transfection with *BRCA1*. Standard deviations are presented in *A* and *B*. At least 100 β-gal-positive cells were analyzed in three fields for each transfection in two independent experiments.

overexpressed state. Overexpression was obtained by cleaving the pcDNA3-BRCA1 plasmid at the unique *Pvu*I site within the ampicillin resistance gene (Fig. 2*A*). As a negative control, the same plasmid was digested with *Hin*dIII, which is located between promoter and the ORF, and *Afl*II, which cuts the plasmid within the *BRCA1* gene at nucleotide 1538. This double cleavage eliminates the likelihood of recreating the intact expression vector if the plasmid incorporates as tandem repeats in the appropriate orientation. The expression of BRCA1 protein after transfection with DNA linearized with *Pvu*I, but not double-digested with *Hin*dIII–*Afl*II, was confirmed by transfection of Cos7 cells (Fig. 2*B*). By using this approach, overexpression of BRCA1 protein again resulted in reduction of colony number when transfected into the U2OS cell line but not into the HBL100 cell line (Fig. 2*C*).

Inactivation of pRb with the E7 Oncoprotein Abrogates BRCA1-Mediated Growth Suppression. The human papilloma virus oncoprotein, E7, binds to and inactivates pRb. To prove that the presence of functional Rb protein is critical for growth suppression by BRCA1, we produced U2OS cells stably expressing E7 protein and transfected them with pcDNA3-BRCA1 vector digested with either *Hin*dIII–*Afl*II or *Pvu*I as described above. We found that in the presence of E7 protein, BRCA1 was not able to suppress cell growth (Fig. 2*C*).

***BRCA1* Suppresses Growth of *rb*+/+, but Not *rb*−/−, Mouse Embryo Fibroblasts.** Our results show that BRCA1 growth arrest depends on the presence of functional Rb protein. These results are based on the study of immortal cell lines that may accumulate multiple unknown mutations during propagation *in vitro*. Although the inactivation of pRb protein by coexpression with HPV-E7 also confirms the importance of Rb for BRCA1-induced growth arrest, the E7 protein also inhibits other members of the Rb family, p107 and p130 (15, 16). To resolve these questions, we used isogenic cells derived from mouse embryo fibroblasts with targeted disruption of the *rb* gene, as well as primary fibroblasts derived from mouse embryos heterozygous for *rb* deletion, and compared them with cells from normal embryos. These primary fibroblasts (gift of Tyler Jacks) were cotransfected with the *lacZ* expression vector (as a transfection marker) and an excess of a *BRCA1*-containing plasmid or vector control and assessed for BrdUrd uptake as a marker of cell proliferation (Fig. 3). When the *BRCA1* gene was overexpressed in Rb+/+ mouse embryo fibroblasts, we observed a 45% decrease in the number of proliferating cells compared with transfection with vector alone. In Rb+/− cells, the decrease in proliferating cells was 22%, and in Rb−/− cells, BRCA1 overexpression did not affect BrdUrd uptake. These results therefore confirm that the presence of the Rb protein, and not Rb-related proteins such as p107 and p130, is important for the *BRCA1*-induced growth arrest.

BRCA1 Growth Suppression Is p53 Independent. It has been shown that, in the presence of p53, BRCA1 protein can coactivate transcription of p53-responsive elements (11, 12). Therefore, we originally expected that not only would Rb status affect *BRCA1*-imposed growth suppression but also the absence of p53 might have an impact on *BRCA1* growth effects. However, several cell lines in our panel (Fig. 1) had a normal *Rb* and a mutated p53 gene (HaCaT and UNC7), and both of these cell lines showed suppression of colony formation when *BRCA1* was transfected. To further confirm that *BRCA1* can suppress cell growth in the absence of functional p53, we partially inactivated p53 in U2OS cell line by overexpression of temperature-sensitive mutant of

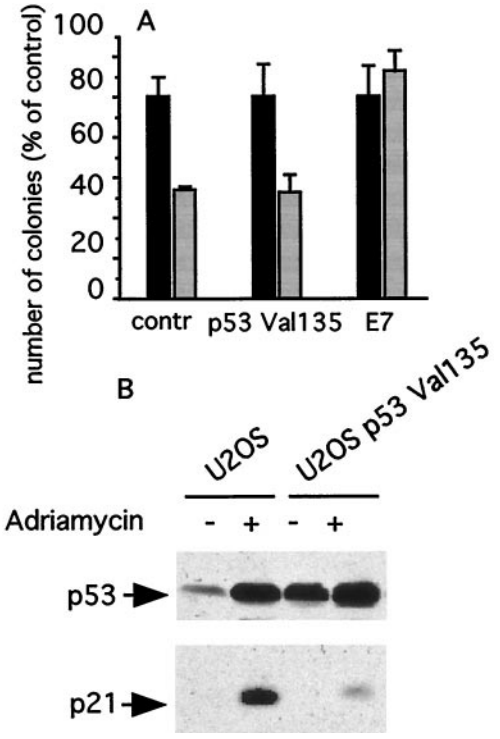

Fig. 4. *BRCA1* overexpression in cells with compromised p53 or pRb. (*A*) Colony formation assay of U2OS control cells or U2OS overexpressing p53Val135 or E7 was performed as in Fig. 1. ■, transfection with vector alone; ▨, *BRCA1* transfection. (*B*) Western blot analysis of p53 and p21 proteins induction 18 h after treatment with Adriamycin.

p53 (p53Val135), which at 37–39°C is acting as a dominant negative (17). To prove that p53 function was compromised, the clone of U2OS cells expressing the highest amount of mutant p53 was treated with Adriamycin, and the induction of p53 and p21 proteins was analyzed by Western blotting (Fig. 4*B*). From 18 to 24 h after treatment, the control cells showed induction of p53 and p21 proteins as compared with untreated cells. However, the p53val135 clone had almost unchanged levels of p53 and much lower induction of p21 protein than control cells. When used to assess colony formation assay after *BRCA1* transfection, the p53val135 clone showed the same growth suppression as the parental U2OS cell line. By contrast, U2OS cells expressing the E7 protein showed no inhibition of colony formation (Fig. 4*A*).

Adenovirus-Mediated *BRCA1* Overexpression Induces Accumulation of Cells in G_1 Phase of the Cell Cycle. To confirm our observations by using other measures of growth inhibition, we infected U2OS and HBL100 cell lines with adenovirus bearing the *BRCA1* or *GFP* (used as control) genes. Construction and characterization of these adenoviruses will be published elsewhere (M.C. and R.A.J., unpublished work). Approximately the same level of BRCA1 protein induction (≈2- to 3-fold) was found for both cell lines as measured by Western blotting on the second day after infection. The status of cell proliferation was monitored by flow cytometric analysis. We found that *BRCA1* induced accumulation of U2OS cells in G_0/G_1 phase of cell cycle, compared with the *GFP* control, 3 days after infection (Fig. 5). This increase in G_0/G_1 subpopulation was more obvious when cells were treated with the mitotic inhibitor nocodazole, which uncovers cells arrested at G_1. No inhibition of cell growth was found when the HBL100 cell line was infected with the Adeno-BRCA1 virus. Taken together, these data show that enforced overexpression of

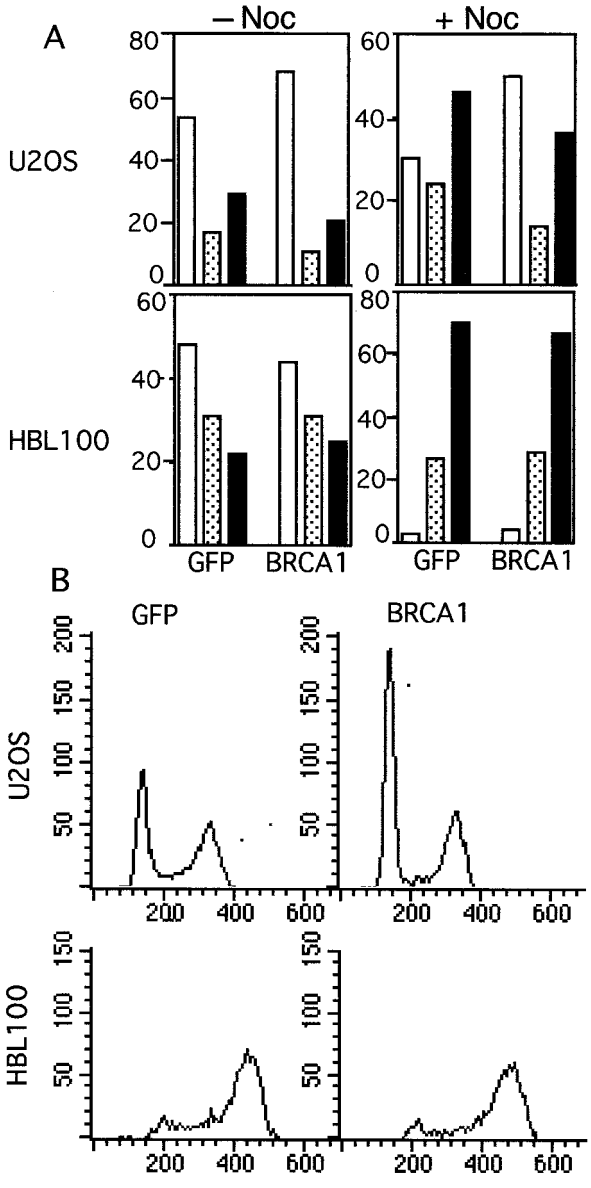

Fig. 5. Overexpression of *BRCA1* results in accumulation of cells in G_0/G_1. Cells were infected with recombinant adenovirus bearing either *BRCA1* or *GFP* gene as control and analyzed by flow cytometry 3 days after infection. (*A*) Cell cycle distribution of U2OS and HBL100 cells with or without nocodazole (*Noc*) treatment. □, percentage of cells in G_0/G_1 phase of cell cycle; ▨, percentage of cells in S phase of cell cycle; ■, percentage of cells in G_2/M phase of cell cycle. (*B*) DNA histogram is shown for nocodazole-treated cells described in *A*. At least three experiments were performed, and a representative experiment is shown.

BRCA1 induces a G_1 arrest and reduced colony formation in cells that have an intact pRb.

Interaction of *BRCA1* and pRb *in Vitro*. Our finding that *BRCA1* can exert its effect on cell growth through Rb protein prompted us to investigate whether interaction between these two proteins may occur. Indeed, we first found that *in vitro*-translated full-length BRCA1 protein was able to bind GST-Rb ABC (amino acids 379–928) and not GST-Rb C (amino acids 780–928) or the GST protein alone (Fig. 6*B*). To localize more precisely the region of *BRCA1* involved in the interaction with pRb, a series of truncated BRCA1 fragments were tested. The NH_2-terminal 394 aa (ScC7 fragment, amino acids 1–394) also showed stable

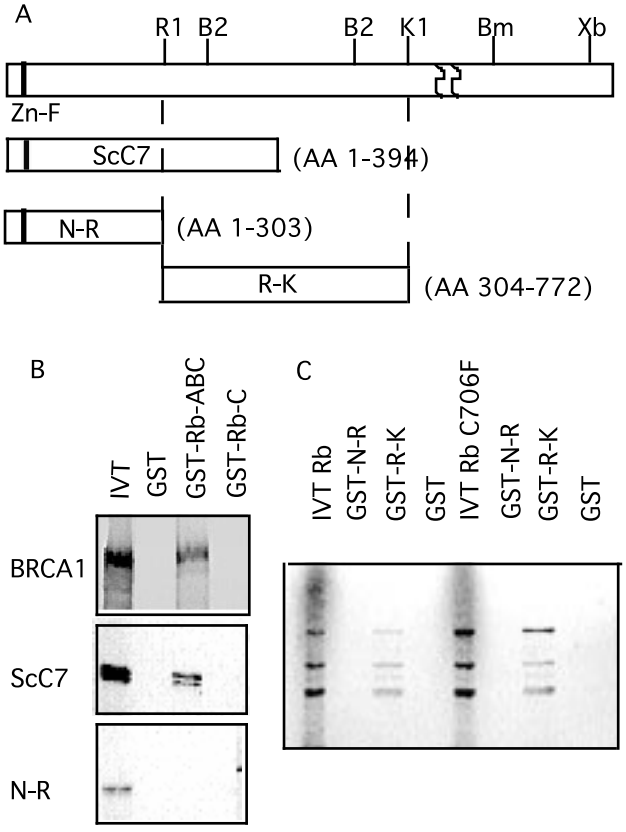

Fig. 6. Identification of BRCA1-Rb interactions. (*A*) Schematic representation of BRCA1 fragments used for *in vitro* translation or GST-fusion proteins. (*B*) *In vitro*-translated [^{35}S]methionine-labeled BRCA1 fragments were incubated with GST–Rb recombinant proteins or GST alone. The complexes were collected with glutathione–Sepharose and resolved on SDS/PAGE followed by autoradiography. (*C*) *In vitro*-translated Rb or mutated Rb (C706F) proteins were used in binding to GST–BRCA1 fragments as described in *B*. One-fifth of the input was loaded in the IVT lane.

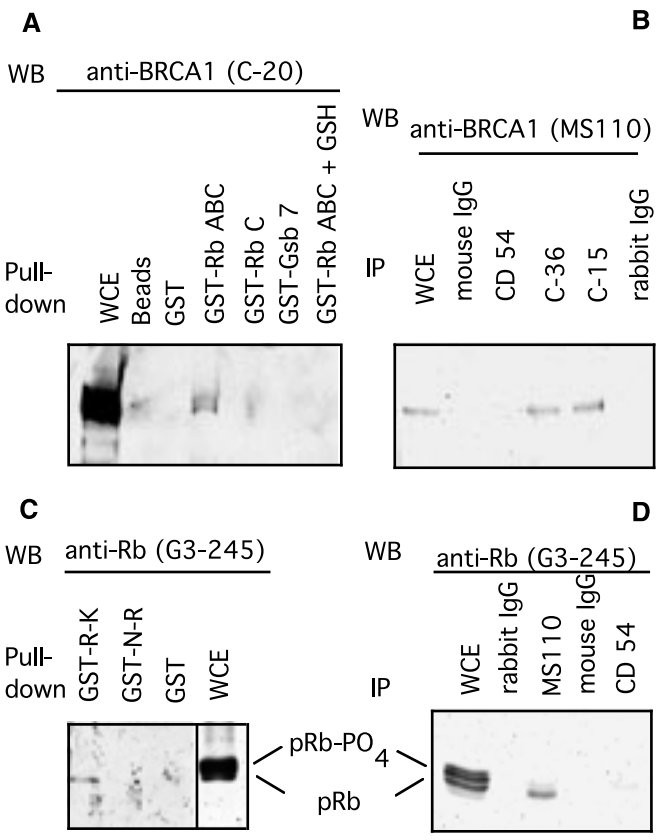

Fig. 7. Rb interacts with BRCA1 protein. (*A* and *C*) Pull-down assay with the use of recombinant GST–Rb or GST–BRCA1 polypeptides. GST fusion proteins were incubated with U2OS (*A*) or SK-N-SH (*C*) cell lysates, and bound BRCA1 or Rb proteins were identified by Western blot (WB). Protein (100 μg) was loaded into WCE lanes. Approximately 1 mg of protein was used for each pull-down reaction. In *A*, GST-Grb7 protein was used as a negative control; in the last lane, the GST–RbABC/BRCA1 protein complex was released from the glutathione–Sepharose with glutathione (GSH) before loading as described in *Methods*. (*B* and *D*) Coimmunoprecipitation of pRb and BRCA1. Rb or BRCA1 proteins were immunoprecipitated from U2OS cell lysate by using anti-Rb (C-36 or C-15) (*B*) or anti-BRCA1 (MS110) (*D*). Normal mouse or rabbit IgG or anti-CD54 antibody was used as a negative control. Total protein (100 μg) was loaded into *WCE lanes* and $\approx$ 2 mg of protein was used for each immunoprecipitation.

binding to GST-Rb ABC protein (Fig. 6*B*). However, further truncation of BRCA1 (N-R, amino acids 1–303) completely eliminated the interaction with GST-Rb ABC.

On further analysis of the sequences within this 90-aa putative Rb binding domain, we found an Rb binding motif, LxCxE at amino acids 358–362. However, when the LxCxE was mutated to LxPxE or when the pentapeptide was removed by an in-frame deletion, the BRCA1 binding domain was still able to interact with pRb (data not shown).

To further demonstrate the binding of pRb to BRCA1, we reversed the probe and target proteins by using GST fusion proteins with BRCA1 fragments GST-N-R (amino acids 1–303) and GST-R-K (amino acids 304–772 shown in Fig. 6*A*) as probe proteins and pRb as target. As before, *in vitro*-translated Rb protein bound the GST-R-K fragment containing previously identified pRb binding domain in exon 11, but not with the GST-N-R protein or GST alone (Fig. 6*C*). A mutated Rb protein carrying a C706F missense mutation which does not bind to oncoproteins E1A and large T antigen but does bind to c-*myc* and L-*myc* (18) also bound to GST-R-K (Fig. 6*C*).

BRCA1 Protein Binds to the Hypophosphorylated Form of pRb. To demonstrate that pRb can pull down intact BRCA1, we incubated recombinant GST–Rb proteins with U2OS cellular lysate and found that only the Rb ABC protein was able to pull down endogenous BRCA1 (Fig. 7*A*). Extending this analysis, we showed that BRCA1 and pRb can be coimmunoprecipitated from cell extracts by using anti-Rb antibodies (Fig. 7*B*). In growing cells, Rb protein is usually present as multiple bands with the faster migrating form representing the hypophosphorylated protein and the slower migrating forms that are differentially phosphorylated. To identify which species of pRb binds preferentially to BRCA1, whole cell extracts of SK-N-SH human neuroblastoma cell line were incubated with the recombinant GST-BRCA1 proteins described in Fig. 6. Our results show that only the hypophosphorylated form of pRb affinity precipitates with GST-R-K fragment (Fig. 7*C*). Similar results were obtained when the complex was immunoprecipitated from U2OS cells (Fig. 7*D*) by using an anti-BRCA1 antibody.

Deletion of Amino Acids 303–394 from BRCA1 Protein Inactivates Its Growth-Suppressive Property. To test the biological role of Rb-BRCA1 interaction, we performed an in-frame deletion of the 92 aa (amino acids 303–394) from BRCA1 which comprised the putative binding region for Rb protein. When used in colony formation assay, BRCA1-delta90 had no effect on growth control (Fig. 8). A construct generating the COOH-terminal

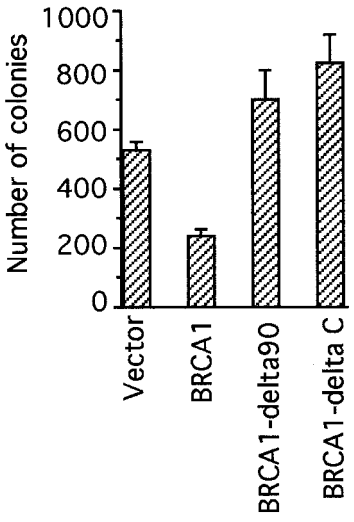

Fig. 8. A mutant BRCA1 protein bearing a deletion of the Rb binding domain does not suppress cell growth. Colony formation assay was performed in U2OS cells (as in Fig. 1) with wild-type BRCA1 or BRCA1 bearing an in-frame deletion of 90 aa (amino acids 303–394) or a COOH-terminal truncation. Each transfection was done in triplicate in three independent experiments.

truncation of BRCA1 was also ineffective in inhibiting colony formation.

Discussion

We have found that the ability of BRCA1 to suppress growth depends on the presence of a functional Rb protein. Although pRb may be required for BRCA1-mediated growth arrest, we recognize that this, in itself, is not evidence that the direct biochemical interaction of the two proteins is responsible for BRCA1 growth inhibition. pRb serves as a downstream effector in several pathways associated with growth suppression, and BRCA1 could presumably act upstream of pRb in any of these pathways, requiring pRb only as the final acceptor of the negative growth signal.

One intriguing candidate for downstream effector of *BRCA1* growth inhibition appeared to be $p21^{Waf1/Cip1}$. Evidence exists showing that *BRCA1* can activate the transcription of p21 even in the absence of p53, suggesting a p53-independent pathway (19). Our data show that this cannot be the only mechanism for *BRCA1*-induced growth inhibition. First, no p21 protein induction was observed at low levels (2- to 5-fold) of *BRCA1* overexpression achieved in transfection of U2OS cells (data not shown). Second, p21 induction blocked cell proliferation of Rb negative SAOS-2 cells (20). Thus, we believe that at levels of *BRCA1* overexpression usually achieved in transfection experiments, *BRCA1* uses pathways other than a p21-mediated mechanism to block cell proliferation.

While the manuscript was in preparation, the association of *BRCA1* with components of the histone deacetylase complex was reported (21). This finding provides yet another possible explanation of how *BRCA1* growth arrest may depend on Rb. The complex of Rb with histone deacetylase is thought to suppress transcription of E2F-responsive genes. *BRCA1* may target this complex to specific genes regulated by progression through the cell cycle or after DNA damage or differentiation. The inhibition of estrogen receptor signaling by overexpressed *BRCA1* as recently reported (22) also supports this hypothesis.

The *BRCA1*/*Rb* interaction may have interesting clinical ramifications. If pRb is a biochemical modulator of *BRCA1* action, then it is conceivable that reductions in the level of pRb expression in breast epithelia may induce a *BRCA1* "null" phenocopy only in *BRCA1* carriers with potentially reduced *BRCA1* expression. In this case, environmental or pharmacological factors that augment pRb expression may serve to delay the onset of cancers in these susceptible women.

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