Previously, we reported that BRCA1 strongly represses the transcriptional activity of estrogen receptor-α (ER-α) in human breast and prostate cancer cells but only weakly inhibits ER-α in cervical cancer cells. We now report that introduction of the human papillomavirus E7 or E6 oncogenes into human papillomavirus-negative cancer cells rescues the BRCA1 repression of ER-α activity and that the E7 and E6 oncoproteins interact directly with BRCA1 in vitro and associate with BRCA1 in vivo in cultured cells. This interaction involves at least two contact points on BRCA1, one within an N-terminal site shown previously to interact with ER-α and the other in a C-terminal region of BRCA1 containing the first BRCA1 C-terminal domain. Point mutations within the zinc finger domains of E7 and E6 inactivated the binding to the N terminus of BRCA1 and reduced their ability to rescue BRCA1 inhibition of ER-α. E6 and E7 also antagonized the ability of BRCA1 to inhibit c-Myc E-box-mediated transactivation and human telomerase reverse transcriptase promoter activity, in a manner dependent upon the zinc finger domains. Finally, the ability of E6 and E7 to antagonize BRCA1 did not involve proteolytic degradation of BRCA1. These findings suggest functional interactions of BRCA1 with E7 and E6. The potential significance of these findings is discussed.

Mutations of the breast cancer susceptibility gene 1 (BRCA1) (chromosome 17q21) are linked to a high risk for breast and ovarian cancers in hereditary early onset breast and breast-ovarian cancer families (1, 2). These mutations also confer and increased risk for these cancer types in Ashkenazi Jewish women unselected for a family history of cancer (3). A large study of cancer risk in BRCA1 cancer families in Europe and North America revealed that BRCA1 mutation carriers are also at significantly increased risk for the development of several other cancer types, including pancreatic cancer, uterine cancer, cervical cancer, and prostate cancer (in men younger than age 65) (4). For cervical cancer, the relative risk of BRCA1 mutation carriers compared with noncarriers was 3.72 (95% confidence interval of 2.26–6.10, p < 0.0001, two-sided test). A subset of patients with sporadic invasive cervical cancer shows hypermethylation of the BRCA1 promoter (5), as do patients with sporadic breast and ovarian cancers (6, 7). BRCA1 promoter methylation may predict a worse prognosis in cervical cancer (8), although this point requires further study. An earlier and smaller study of cancer incidence in the relatives of BRCA1 and BRCA2 mutation carriers revealed about a 4-fold increased risk of cervical cancer in BRCA2-associated families, although the risk in BRCA1 families was not similarly elevated (9). Most interestingly, loss of heterozygosity at chromosome 17q, a site that contains the BRCA1 gene, appears to be a common event in cervical cancer (10).

Previously, we found that the overexpression of BRCA1 inhibits the estrogen (E2)-induced transcriptional activity of the estrogen receptor-α (ER-α) and inhibits E2-stimulated gene expression (11–13). Most unexpectedly, the ability of BRCA1 to repress ER-α activity was found to be cell type-specific. Thus, BRCA1 virtually abolished E2-stimulated ER-α activity in four of four human breast cancer (MCF-7, T47D, MDA-MB-231, and MDA-MB-453) and four of four human prostate cancer (DU-145, LNCaP, PC-3, and TsuPr-1 (now known to be derived from a blander cancer) cell lines; however, BRCA1 caused only a modest or no reduction of ER-α activity in four of four human cervical cell lines (HeLa, SiHa, CaSkii, and C33A) (11). Moreover, BRCA1 overexpression inhibited the E2-stimulated activity of activation function-2, the ligand-inducible transcriptional activation domain of ER-α in breast and prostate cancer cells but not in cervical cancer cell lines (11, 14). Three of the four cervical cancer cell lines that we studied (HeLa, SiHa, and Caski) are known to contain integrated oncogenic human papillomavirus (HPV) genomes: HPV-16 for SiHa and CaSkii and HPV-18 for HeLa (15, 16). The two major HPV oncoproteins, E7 and E6, function in part by inactivating host cell tumor suppressor proteins, retinoblastoma 1 (RB1) (and other retinoblastoma family proteins) and p53 (17). Taken together, these findings raised the possibility that HPV proteins may functionally inactivate BRCA1 in cervical cancer cells.

The purpose of this study was to evaluate the hypothesis that the HPV oncoproteins E6 and E7 can interact with and inactivate the function of BRCA1.

**MATERIALS AND METHODS**

**Cell Lines and Culture**

Human breast cancer (MCF-7 and T47D), prostate cancer (DU-145), and cervical cancer (SiHa, Caski, and HeLa) cell lines and 293T human embryonal kidney cells were obtained from the American Type Culture Collection (Manassas, VA) and cultured as described before (11–14). Briefly, the cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 5 or 10% (v/v) fetal calf serum, l-glutamine (5 mM), nonessential amino acids (5 mM), penicillin (100 units/ml), and streptomycin (100 μg/ml). All cell culture reagents were obtained from BioWhittaker, Walkersville, MD.

**Expression Vectors and Reporters**

**Expression Vectors for GST Proteins**—The GST-E7 (HPV16, wild-type, full-length) expression plasmid was a gift from Dr. Karl Munger.
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(Dept. of Pathology, Harvard Medical School, Boston). The GST-E6 (HPV16, wild-type, full-length) plasmid was a gift from Dr. Peter M. Howley (Dept. of Pathology, Harvard Medical School) (18). The GST-E7-(2–38), -(16–98), and -(38–98) vectors were generated by PCR cloning. Briefly, BamHI and EcoRI digestion sites were designed on the 5’ and 3’ primers, respectively; and the BamHI and EcoRI double digestion products were inserted into BamHI and EcoRI site of the pGEX2T vector (Amersham Biosciences). The GST-E7 C91G expression vector, which encodes a full-length chimeric E7 protein with an inactivating point mutation of the C-terminal zinc finger domain, was provided by Dr. Tony Kouzarides (Wellcome/CR UK Gurdon Institute, Cambridge University, Cambridge, UK) (19). The GST-E6 C66,136G vector, which encodes a full-length chimeric E6 protein with an inactivating point mutation in both zinc finger domains, was generated by PCR cloning, using the pBS-E6 C66,136G plasmid (a gift from Dr. Denise A. Galloway, Fred Hutchinson Cancer Research Center, Seattle, WA (20)) as a template. The BamHI and EcoRI double digestion product was inserted into the BamHI and EcoRI site of pGEX2T vector. Expression vectors for GST-BRCA1 protein fragments corresponding to BRCA1 amino acids 1–324, 260–553, 502–802, 758–1064, 1005–1313, and 1314–1863 were kindly provided by Dr. Toru Ouchi (Ruttenberg Cancer Center, Mount Sinai School of Medicine, New York). These constructs have been described earlier (21).

Expression Vectors for In Vitro Translation and/or Expression within Mammalian Cells—The wild-type BRCA1 expression vector (wtBRCA1) was created by cloning the BRCA1 cDNA into the pcDNA3 vector (Invitrogen) by using artificially engineered 5’ HindIII and 3’ NotI sites (22). cDNAs for BRCA1-(1–1313), -(1–771), -(1–302), (34–300), (67–300), (101–300), and (134–300) within the pcDNA3 or pCMV-Tag2 vector (Stratagene, La Jolla, CA) have been described earlier (12, 23, 24). The BRCA1-(1–320) cDNA fragment plasmid was generated by PCR cloning, and the BamHI and XbaI double digestion product was inserted into the BamHI and XbaI site of the pcDNA3 vector. The BRCA1-(310–806), -(802–1314), -(1314–1863), and -(1532–1749) cDNA fragments were generated by PCR cloning, followed by BamHI and EcoRI double digestion and insertion into the BamHI and EcoRI site of pcDNA3 vector. The pcDNA3 FLAG-E7 expression vector was generously provided by Dr. Tony Kouzarides. The pcDNA3-E6 expression vector has been described earlier (25). The p3XFLAG-E6 and p3XFLAG-E6-(C66,136G) expression vectors were generated by PCR cloning of the FLAG-E6 cDNAs, followed by HindIII and XbaI double digestion and insertion into the HindIII and XbaI site of the p3XFLAG vector (Sigma). The FLAG-E7-(C91G) expression plasmid was created in a similar manner. The correct insertion of the cDNAs was confirmed by sequencing of each of the subcloned plasmids. The ER-α expression vector pCMV-ER-α was used to express ER-α in transient transfection assays of estrogen receptor transcriptional activity (12).

Expression vectors encoding the wt adenovirus E1A-(243R) protein and various mutant or truncated E1A-(243R) proteins (including single point mutations RG2 and 928m; the double point mutants RG2/928m and YH47/928m; and the deletion mutants Δ15–35 and Δ38–67) were provided by Dr. Richard G. Pestell (Lombardi Comprehensive Cancer Center, Georgetown University, Washington, D. C.) (26). Expression vectors encoding the wild-type SV40 large T oncoprotein (SV40T) and a mutant protein defective for RB family protein binding (mut-SV40T) (27) were also provided by Dr. Pestell.

Reporters—The estrogen-responsive reporter ERE-TK-Luc is composed of the vitellogenin A2 estrogen-responsive enhancer (ERE), controlling a minimal thymidine kinase promoter (TK81) and luciferase, in plasmid pGL2 (28). Assays of ER-α transcriptional activity utilizing the ERE-TK-Luc reporter are described below. The E-box-Luc reporter contains a canonical c-Myc E-box upstream of a minimal promoter and luciferase (29). Its activity is stimulated by c-Myc and inhibited by the c-Myc/Max repressor Mad1 (29). The hTERT-Luc reporter consists of the core human telomerase reverse transcriptase (hTERT) promoter upstream of the luciferase gene, within the pGL3 plasmid (29).

Assays of Estrogen-dependent Transcriptional Activity

Subconfluent proliferating cells in 24-well dishes were incubated overnight with 0.25 μg of each indicated vector in serum-free DMEM containing LipofectamineTM (Invitrogen). The total transfected DNA was kept constant by addition of the appropriate control vectors. The cells were washed, incubated in phenolphtalein-free DMEM containing 5% charcoal-stripped serum (obtained from the Tissue Culture Core Facility of the Lombardi Comprehensive Cancer Center) (0.2 ml per well) ± 17β-estradiol (E2, 1 μM or 10 nM, as indicated) for 24 h, and harvested for luciferase assays. To control for transfection efficiency, plasmid prSV-β-gal was co-transfected to allow normalization of luciferase values to β-galactosidase activity in the same sample. Values are means ± S.E. of four replicate wells and are representative of two or more independent experiments.

Immunoprecipitation

To study the association of the endogenous BRCA1 and oncoprotein E7, proliferating SiHa cells at about 80% of confluenced in 100-mm plastic dishes were harvested; and whole cell extracts were prepared as described below (12, 14, 24), using RIPA buffer. Each IP was carried out as described before by using 10 μg of antibody (anti-BRCA1 (I-20, Santa Cruz Biotechnology, Santa Cruz, CA), anti-E7 (ED-17, Santa Cruz Biotechnology), or nonimmune rabbit or mouse IgG (negative control)) and 500 μg of total cell extract protein. The precipitated proteins were collected using protein A/G Plus-agarose beads (Santa Cruz Biotechnology) and eluted using boiling Laemmli sample buffer. The eluted proteins were then subjected to Western blotting to detect the BRCA1 or E7 proteins, as described below.

To study the interaction of BRCA1 with E6, subconfluent proliferating 293T cells were transfected overnight with the p3XFLAG-E6 and wtBRCA1 expression vectors (see above) using LipofectamineTM, washed, post-incubated for 24 h to allow gene expression, harvested, and then immunoprecipitated using anti-BRCA1 (as above) or nonimmune rabbit IgG (control). The precipitated proteins were collected using protein A/G Plus-agarose beads or anti-FLAG M2 affinity gel (Sigma), washed, eluted, and subjected to Western blotting to detect the BRCA1 protein or the FLAG epitope (see below).

Western Blotting

Western blotting was carried out as described before (23, 24). The IPs (see above) were electrophoresed on a 4–12% SDS-polyacrylamide gradient gel, transferred to nitrocellulose membranes (Millipore), and blotted using primary antibodies directed against BRCA1 (C-20, rabbit polyclonal, Santa Cruz Biotechnology, 1:200); E7 (TVG710Y, Santa Cruz Biotechnology), or the FLAG epitope (M2, mouse monoclonal antibody, Sigma, 1:500 dilution). As a control, nonprecipitated lysates (50 μg of cell protein) were blotted on the same gels. The blotted protein bands were visualized using the enhanced chemiluminescence (ECL) detection system (Amersham Biosciences), with colored markers (Bio-Rad) as size standards.

In a separate experiment, cells were transfected overnight with wild-type or mutant FLAG-E6 or FLAG-E7, post-incubated for 24 h to allow gene expression, and Western-blotted (50 μg of total cell protein per lane) to detect BRCA1 (C-20 antibody), E6 protein (anti-HPV16/18 E6,
GST Capture Assays

GST bead assays were performed essentially as described earlier (12, 14, 24). In vitro translated (IVT) proteins were prepared by in vitro transcription and translation, using the T7 promoter of the pcDNA3 vector or the T3 promoter of the pCMV-Tag2B vector. The proteins were labeled using [35S]methionine (Amersham Biosciences) or Transend™ tRNA (Promega Corp., Madison, WI), and in vitro transcription-translation was carried out using the TntT-coupled rabbit reticulocyte lysate system (Promega), according to the manufacturer’s instructions. The GST fusion proteins were generated from cDNAs cloned into the pGEX2T vector, expressed in E. coli, and purified by affinity chromatography using glutathione-Sepharose (Amersham Biosciences). The source or construction of the expression vectors for various GST-BRCA1, GST-E7, and GST-E6 proteins is described above. Labeled IVT proteins were incubated with GST protein (negative control) or GST fusion proteins for 4 h at 4 °C, recovered using GSH-agarose beads, eluted in boiling sample buffer, and analyzed by SDS-PAGE autoradiography. In all GST capture assays, the IVT input lanes show 10% of the protein quantity used in the GST capture assays. All experiments included a lane corresponding to capture by beads coated with GST alone, as a negative control. The GST fusion proteins were visualized by Western blotting, using anti-GST mouse monoclonal antibody 27–4577–01 (Amersham Biosciences, 1:5000). Additional details relevant to specific experiments are provided in the text or figure legends.

Knockdown of BRCA1 Using siRNAs

The BRCA1 and corresponding control (scrambled sequence) siRNAs have been described earlier (29). All siRNAs were chemically synthesized by Dharmacon, Inc. The sequences used to synthesize the siRNAs were as follows: BRCA1 siRNA, 5′-AATGCGAAAATGCTAATGTA-3′, and control siRNA, 5′-CGATAGATACACAGATTGAAT-3′.

For siRNA treatments, subconfluent proliferating cells were transfected with 50 nM of siRNA using the siPORT Amine transfection reagent (Ambion). The maximal reduction of protein levels required a 72-h incubation with the siRNA. Prior studies established that under these conditions, none of the siRNAs caused cytotoxicity, based on cell morphology and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays.

Statistical Methods

Where appropriate, statistical comparisons were made using the two-tailed Student’s t test.
RESULTS

Rescue (Reversal) of the BRCA1-mediated Repression of ER-α Transcriptional Activity by HPV Oncoproteins E7 and E6—We tested the ability of several different DNA tumor virus-encoded transforming oncoproteins to rescue (reverse) the BRCA1-mediated repression of ER-α activity, including HPV oncoproteins E6 and E7, the adenovirus E1A oncoprotein, and the SV40 large T protein. Schematic diagrams showing the domain structure of these proteins are provided in Fig. 1A. We utilized a standard transient transfection assay of ER-α transcriptional activity using an estrogen-responsive reporter, ERE-TK-Luc (12, 14, 24). As illustrated in Fig. 1B, in the absence of E7 or E6, exogenous wtBRCA1 reduced the estrogen (E2)-stimulated reporter activity to less than 1% of the control value ($p < 0.001$, two-tailed $t$ test) in human breast (MCF-7 and T47D) and prostate (DU-145) cancer cells, whereas the empty pcDNA3 vector had little or no effect on the reporter activity. In contrast, expression vectors encoding the E7 and E6 oncoproteins from HPV-16 (an oncogenic HPV strain) rescued the wtBRCA1-mediated repression of ER-α activity in HPV-negative human breast and prostate cancer cell lines (Fig. 1B).

Besides E7 and E6, several other DNA viral oncoproteins were also able to rescue the BRCA1 inhibition of ER-α transcriptional activity. Thus, in studies of DU-145 human prostate cancer and T47D breast cancer cells, the adenovirus E1A-(243R) and SV40 large T oncoproteins also rescued the BRCA1 inhibition of ER-α activity ($p < 0.001$) (Fig. 1, C and D, respectively). The E1A-(243R) transforming protein is the product of an alternatively spliced mRNA, the full-length form of which encodes the E1A-(289R) protein (30). E1A-(243R) differs from E1A-(289R) in that E1A-(289R) is missing a conserved region (CR3) that is present in E1A-(289R). Studies of a small series of mutant E1A-(243R) genes revealed that expression vectors encoding proteins with an N-terminal mutation or deletion (RG2, Δ38–67, RG2/928m, and Δ15–35) failed to rescue the BRCA1 repression, whereas those containing several other mutations (928m and YH47/928m) retained the ability to reverse the BRCA1-mediated repression of ER-α activity ($p < 0.001$) (Fig. 1C). E1A-(928m) encodes a mutant protein with a defective retinoblastoma (RB) protein binding domain, suggesting that the RB binding domain is dispensable for the rescue of BRCA1-mediated repression. Expression vectors encoding wild-type SV40 large T and a mutant with a defective RB binding domain both rescued the BRCA1 repression ($p < 0.001$) (Fig. 1D), again suggesting that interaction with RB family proteins (RB1, p107, and p130) is not required for rescue.

For reference, Fig. 1E shows the effect of wtBRCA1 on ER-α signaling in three human cervical cancer cell lines, each of which contains an oncogenic HPV genome (HPV-16 or HPV-18) as follows: CaSkI, SiHa, and HeLa. As compared with the HPV-negative breast and prostate cancer cell lines for which wtBRCA1 caused a reduction in estrogen-stimulated ER-α activity to close to the basal levels observed in the absence of estrogen (2–3-log reduction), wtBRCA1 only had a modest effect in the cervical cancer cells, reducing ER-α activity by only 0–40% (well under a 1-log reduction). Our previous studies indicate that wtBRCA1 is well expressed in these cell lines (14).

Direct Interaction of BRCA1 with the HPV-E6 and HPV-E7 Oncoproteins and Mapping of Interacting Sites—The findings described above suggest that several different DNA viral transforming oncoproteins are capable of inactivating at least one function of BRCA1, i.e. its ability to inhibit ER-α activity. Because E1A and SV40 large T cells have not been implicated in human carcinogenesis, we chose to further investigate the mechanism(s) by which HPV-E6 and HPV-E7 can inactivate BRCA1 function.

GST-E7 and GST-E6 Proteins Capture C-terminally Truncated BRCA1 Proteins—We used GST capture assays to determine whether BRCA1 can interact physically with the E7 or E6 proteins. The structure of the BRCA1 proteins used in these assays is illustrated schematically in Fig. 2A. Full-length GST-E7 and GST-E6 proteins were expressed in E. coli, and the expression of these GST fusion proteins was confirmed by Western blotting, using an anti-GST antibody (Fig. 2B). Fig. 2C shows the expression of various 35S-labeled IVT BRCA1 proteins, as visualized by SDS-PAGE autoradiography. GST capture assays revealed that the full-length (1863 amino acids) BRCA1 protein was captured by beads coated with GST-E7 or GST-E6 but not by beads coated with GST alone (negative control) (Fig. 2D). In addition to the full-length BRCA1, several IVT C-terminally truncated BRCA1 proteins (BRCA1-(1–1313), (1–771), and (1–302)) were also captured by GST-E7 and by GST-E6 (Fig. 2E). These findings suggest a physical interaction of BRCA1 with the E7 and E6 proteins. They also suggest the presence of a contact point within the N terminus of BRCA1.

Capture of Different BRCA1 Protein Fragments by Full-length GST-E7 and GST-E6—To further delineate the binding site(s) for E7 and E6 on the BRCA1 protein, we tested the ability of GST-E7 and GST-E6 to capture different IVT BRCA1 fragments across the full length of the BRCA1 protein and within the N-terminal amino acids 1–302 (see Fig. 3A). Studies of four protein fragments spanning the length of BRCA1 revealed an interaction between the N- and C-terminal BRCA1 proteins (amino acids 1–302 and 1314–1863) with both E7 (Fig. 3B) and E6 (Fig. 3C). In each case, GST alone failed to capture any of the IVT BRCA1 proteins. Fig. 3, D and E, shows the ability of E7 and E6, respectively, to capture each of four different IVT N-terminal BRCA1 fragments. Whereas BRCA1-(34–300) and (67–300) were captured by both GST-E7 and GST-E6, neither E7 nor E6 captured BRCA1-(101–300) and (134–300). These findings suggest that the BRCA1 N-terminal Ring domain (amino acids 20–64) is not required for the interaction with E7 or E6, but the interaction does require amino acids 67–100. Finally, BRCA1-(1532–1749), which contains the first BRCA1 C-terminal repeat (BRCT) domain, was sufficient to mediate an interaction with E7 and with E6 (Fig. 3F); however, we cannot rule out the possibility of an additional contact point(s) for E7 or E6 within amino acids 1314–1863 of BRCA1.

Capture of Full-length IVT E7 and E6 by Different GST-BRCA1 Proteins—To substantiate further the validity of the BRCA1-E7 and BRCA1-E6 interactions, we performed the reverse capture experiment, i.e. we tested the ability of a set of overlapping GST-BRCA1 proteins to capture the full-length IVT E7 protein. The construction of these GST-BRCA1 expression vectors has been described earlier (21). Consistent with our previous results, both N-terminal (amino acids 1–324) and C-terminal (amino acids 1314–1863) BRCA1 proteins captured full-length IVT E7 (Fig. 4A). The ability of different GST-BRCA1 proteins to capture full-length IVT E6 is shown in Fig. 4B. Here again the C-terminal fragment (amino acids 1314–1863) and the N-terminal fragment (amino acids 1–324) showed significant capture of IVT E6, whereas several other fragments showed a much smaller degree of capture. The expression of the different GST-BRCA1 proteins, visualized by anti-GST Western blotting, is shown in Fig. 4C. In some cases, smaller bands, which may reflect degraded GST-BRCA1 proteins or cross-reacting species, were observed. The correct bands are indicated in the figure. This Western blot is typical of a number of repeat experiments. Taken together, we can demonstrate using bi-directional GST capture assays that E7 and E6 each interact with N- and C-terminal domains within BRCA1.
Association of the BRCA1 Protein with the E7 and E6 Oncoproteins in Cultured Cells—To determine whether BRCA1 can interact with the E7 and E6 oncoproteins in vivo, we performed two-way IP-Western blots using SiHa cells, a human cervical cancer cell line with an integrated HPV-18 genome (31). Here, IP of endogenous E7 using an anti-E7 antibody co-precipitated both the E7 and BRCA1 proteins, whereas a control IP using an equal quantity of nonimmune antibody failed to precipitate either protein (Fig. 5A). Conversely, an anti-BRCA1 antibody (but not a control nonimmunoglobulin antibody) co-precipitated the endogenous BRCA1 and E7 proteins (Fig. 5B). As expected, unprecipitated SiHa lysates exhibited both BRCA1 and E7, whereas cell lysates prepared from a breast cancer cell line (MCF-7) contained BRCA1 but not E7. We lysates exhibited both BRCA1 and E7, whereas cell lysates prepared from a breast cancer cell line (MCF-7) contained BRCA1 but not E7. We had difficulty detecting the endogenous E6 protein in SiHa cells. Thus, to evaluate the BRCA1-E6 interaction, we transfected 293T embryonal kidney cells with a FLAG-tagged E6 expression vector and a wtBRCA1 vector (to increase the levels of BRCA1 and facilitate detection of the interaction). In this study, a BRCA1 IP was found to co-precipitate the BRCA1 and FLAG-E6 proteins (Fig. 5C), whereas an anti-FLAG IP co-precipitated FLAG-E6 and BRCA1 (Fig. 5D). In both cases, the control IP failed to precipitate E6 or BRCA1. These findings suggest that the BRCA1 protein can associate with the E7 protein and with the E6 protein in vivo.

Interaction of BRCA1 with Truncated and Mutant E7 and E6 Proteins—To elucidate further the BRCA1-E6/E7 protein interactions, we tested the ability of several truncated or mutant GST-E7 and GST-E6 proteins to capture IVT N-terminal BRCA1 proteins. Diagrams showing the domain structure of the GST-E7 proteins utilized in this study are provided in Fig. 6A. Fig. 6B shows the expression of five different GST-E7 proteins, and Fig. 6C and D shows the ability of these GST-E7 proteins to capture IVT BRCA1-(1–302) or -(1–320). These data suggest that the binding of E7 to the N terminus of BRCA1 is mediated by the C-terminal region of E7 (amino acids 38–98) and does not require the N terminus of E7 (amino acids 1–38). They also indicate that a point mutation that disrupts the zinc finger domain of E7((C91G)) also disrupts the binding of E7 to BRCA1.

Fig. 6, E and F, shows the domain structure and expression, respectively, of five different GST-E6 proteins used to study the interaction between E6 and the N terminus of BRCA1 (amino acids 1–302). The GST capture experiment revealed no capture of BRCA1 by an N-terminal fragment of E6 (amino acids 2–40), whereas amino acids 16–83 and 80–151 did mediate capture of BRCA1 (Fig. 6G). Note that amino acids 16–83 and 80–151 contain the N-terminal and C-terminal zinc finger domains, respectively, of the E6 protein. A double point mutation that disrupts both zinc finger domains of E6((C66,136G)) abrogated the binding of E6 to BRCA1. Taken together, these findings suggest that the binding of both E7 and E6 to the N terminus of BRCA1 requires intact zinc finger domains.

Rescue of BRCA1 Repression of ER-α Activity by Wild-type Versus Mutant E6 and E7 Oncoproteins—Based on these findings, we compared the ability of wild-type versus mutant E7 and E6 proteins to rescue the BRCA1-mediated repression of ER-α activity. For these studies, we
**FIGURE 3.** Capture of different IVT BRCA1 protein fragments by full-length GST-E7 and GST-E6. A, domain structure of BRCA1 protein and protein fragments. B, capture of different IVT BRCA1 proteins by full-length GST-E7 protein. The ability of GST-E7 to capture a set of IVT BRCA1 protein fragments spanning the length of the BRCA1 protein was determined. The input lane represents 10% of the IVT protein used in the GST capture assays. C, capture of different IVT BRCA1 proteins by full-length GST-E6 protein. Assays were performed as described in A, except that GST-E6 was used as the bait. D, capture of IVT N-terminal BRCA1 proteins by full-length GST-E7 protein. A group of N-terminal BRCA1 protein fragments was tested for capture by full-length GST-E7 protein. E, capture of IVT N-terminal BRCA1 proteins by full-length GST-E6 protein. A group of N-terminal BRCA1 protein fragments was tested for capture by full-length GST-E6 protein. F, capture of IVT BRCA1 BRCT1 by full-length GST-E7 and GST-E6 proteins. An IVT BRCA1 protein fragment containing the first BRCT1 was tested for capture by the GST-E7 and GST-E6 proteins. NES, nuclear export signal; AD1, activation domain.
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FIGURE 4. Capture of IVT full-length E7 and E6 proteins by various GST-BRCA1 proteins. A, capture of full-length IVT E7 by different GST-BRCA1 proteins. The ability of a set of overlapping GST-BRCA1 proteins to capture in vitro translated full-length E7 was tested. B, capture of full-length IVT E6 proteins by different GST-BRCA1 proteins. The ability of a set of overlapping GST-BRCA1 proteins to capture in vitro translated full-length E7 was tested. C, Western blot showing the expression of different GST-BRCA1 proteins. The different GST-BRCA1 proteins utilized in A and B were visualized by Western blotting using an antibody directed against GST. The correct band is indicated by a box in the figure.

FIGURE 5. In vivo association of BRCA1 and the E6 and E7 proteins. A, IP of endogenous E7 and BRCA1 in SiHa cells. Subconfluent proliferating SiHa human cervical cancer cells were subjected to IP using an anti-E7 antibody and Western-blotted to detect E7 and BRCA1. See “Materials and Methods” for details. B, IP of endogenous BRCA1 co-precipitates E7 in SiHa cells. Subconfluent proliferating SiHa cells were subjected to IP using an anti-BRCA1 antibody and Western-blotted to detect BRCA1 and E7. See “Materials and Methods” for details. C, IP of BRCA1 co-precipitates BRCA1 and exogenous FLAG-tagged E6 in 293T cells. Subconfluent proliferating 293T cells were transfected overnight with FLAG-E6 and wtBRCA1 expression vectors (10 μg of each vector per 100-mm dish) using Lipofectamine™, post-incubated for 24 h to allow gene expression, immunoprecipitated using an anti-BRCA1 antibody, and Western-blotted to detect the BRCA1 and FLAG-E6 proteins. D, anti-FLAG IP co-precipitates exogenous FLAG-E6 and BRCA1 in 293T cells. 293T cells were transfected as described above in C, post-incubated for 24 h to allow gene expression, immunoprecipitated using an anti-FLAG antibody, and Western-blotted to detect the FLAG-E6 and BRCA1 proteins.

FIGURE 6. Effect of E7 and E6 on Other Functional Activities of BRCA1—Previ-ously, we showed that BRCA1 inhibits the activity of a reporter driven by an E-box element (E-box-Luc), the canonical DNA target for the c-Myc transactivation function (29). Here we examined the effect of the E7 and E6 oncoproteins on the ability of BRCA1 to inhibit E box-Luc activity HPV genome-negative MCF-7 and T47D cells. As reported previously (29), wtBRCA1 effectively repressed E-box-Luc activity in both MCF-7 and T47D cells (p < 0.001) (Fig. 8A). Co-expression of wild-type E6 or E7 with wtBRCA1 led to substantially higher E-box-Luc activity than in the absence of E6 or E7 (p < 0.001). Here the ability of E6 to rescue BRCA1 inhibition of E-box activity was somewhat greater than that of E7, but both oncoproteins effected the rescue. We also observed E6- and E7-mediated increases in E-box-Luc activity in the absence of exogenous BRCA1 (p < 0.001). The significance of these findings is considered under “Discussion.”

Previously, we showed that endogenous BRCA1 is present on the TERT promoter and that exogenous wtBRCA1 represses the activity of an hTERT-Luc reporter plasmid (29). The promoter segment in this
FIGURE 6. Capture of BRCA1 by truncated and mutant GST-E7 and E6 proteins. A, schematic diagrams of the truncated/mutant E7 proteins used in this study. B, Western blot showing the expression of truncated and mutant GST-E7 proteins. A set of truncated and mutant GST-E7 proteins were visualized by Western blotting, using an anti-GST antibody. C, capture of IVT BRCA1-(1–302) by truncated and mutant GST-E7 proteins. Different GST-E7 proteins shown in A were tested for their ability to capture an in vitro translated N-terminal portion of BRCA1 (amino acids 1–302). D, capture of IVT BRCA1-(1–771) by truncated and mutant GST-E7 proteins. Different GST-E7 proteins from A were tested for their ability to capture an in vitro translated N-terminal portion of BRCA1 corresponding to amino acids 1–771. E, schematic diagrams of the truncated/mutant E6 proteins used in this study. F, Western blot showing the expression of truncated and mutant GST-E6 proteins. A set of truncated and mutant GST-E6 proteins were visualized by Western blotting, using an anti-GST antibody. G, capture of IVT BRCA1-(1–302) by truncated and mutant GST-E6 proteins. The various GST-E6 proteins shown in D were tested for their ability to capture IVT BRCA1-(1–302).
reporter consists of the core hTERT promoter and contains DNA elements corresponding to c-Myc (E-box), Sp1, and NF-kB-binding sites. The BRCA1-mediated repression of hTERT-Luc activity was probably mediated through the E-box element, because wtBRCA1 did not inhibit Sp1 or NF-kB-inducible promoter activity (29). Here we found that wtBRCA1 effectively repressed hTERT-Luc activity, as described previously, and the activity was (at least partially) rescued by the E6 and E7 oncoproteins (p < 0.001) (Fig. 8B). The E6 rescue was slightly more efficient than that by E7, but both oncoproteins caused a significant increase in hTERT-Luc activity in the presence of exogenous wtBRCA1. As reported before, E6 by itself increased the hTERT promoter activity by severalfold in the absence of exogenous BRCA1 (p < 0.001) (Fig. 8B). E7 also stimulated the hTERT-Luc activity in the absence of wtBRCA1, but to a somewhat lesser extent than did E6. These findings suggest that the ability of E6 and E7 to counteract BRCA1 functional activity is not limited to the inhibition of ER-α signaling.

Neither E6 nor E7 Causes the Proteolytic Degradation of BRCA1—Because the interactions of E6 with p53 and E7 with RB1 are known to result in the proteolytic degradation of these proteins, we tested whether the ability of E6 and E7 to inactivate BRCA1 might be due, in part, to its targeting for degradation. HPV-negative MCF-7 and T47D cells were transiently transfected with expression vectors encoding wt E6, wt E7, or zinc finger defective mutant E6-(C66,136G) or E7-(C91G) and post-incubated for 24 h to allow gene expression. The expression of the E6 and E7 proteins was confirmed by Western blotting (Fig. 9). There was no effect on the BRCA1 protein levels. As positive controls, Western blots revealed that in MCF-7, which has wild-type p53 and RB1, wt E6 caused the loss of p53 protein (Fig. 9A), whereas wt E7 caused the loss of RB1 protein (Fig. 9B). For T47D, which has a single mutant p53 allele (a point mutation that extends the protein half-life) (32), and wild-type RB1, E6 did not cause p53 protein loss (Fig. 9A), but E7 did cause the loss of RB1 protein (Fig. 9B). Note that the zinc finger-disrupted mutants E6 and E7 were well expressed but either failed to cause degradation of p53 or RB1 or were defective in these abilities as compared with wt E6 and wt E7, respectively. These findings suggest that E6 and E7 are competent to cause the degradation of p53 and RB1, respectively, in human breast cancer cells but do not cause degradation of BRCA1.

Zinc Finger Domains of E6 and E7 Are Required for Efficient Rescue of wtBRCA1 Inhibition of hTERT and E-box Promoter Activity—Because the zinc finger domains of E6 and E7 are required for efficient binding to BRCA1 (at least to the N-terminal binding site on BRCA1), we compared the ability of zinc finger-defective mutants of E6 and E7 to rescue the BRCA1 inhibition of hTERT-Luc and E-box-Luc. Consistent with the ERE-TK-Luc assays in Fig. 7A, assays of hTERT (Fig. 10A) and E-box (Fig. 10B) transciptional assay revealed that the zinc finger mutants of E6-(C66,136G) and E7-(C91G) were each severely deficient in their ability to rescue the BRCA1 repression, as compared with the wild-type proteins (p < 0.001, two-tailed t test), although the mutants did retain a small amount of residual activity. These findings suggest that inactivation of BRCA1 repression of ER-α, hTERT, and E-box-Luc activity by the E6 and E7 oncoproteins requires efficient binding to BRCA1, but does not require the proteolytic degradation of BRCA1.
Effect of BRCA1 Knockdown on E6- and E7-mediated Stimulation of hTERT Promoter Activity—Figs. 8 and 10 indicate that in the absence of exogenous BRCA1, wt E6 and wt E7 can stimulate hTERT and E-box promoter activity by about 2.5–4-fold in MCF-7 and T47D cells. These effects could be BRCA1-independent or could be due, in part, to functional inactivation of the endogenous BRCA1 protein. Here we knocked down the endogenous BRCA1 by using a previously validated siRNA (29) to determine the effects of E6 and E7 on hTERT activity in the absence of BRCA1. Consistent with previous findings (29), BRCA1 siRNA alone caused about a doubling of hTERT activity, whereas the control siRNA had little or no effect on hTERT activity (p < 0.001) (Fig. 11A). In the presence of the control siRNA, E6 and E7 caused 2.6–3.2-fold increases in hTERT activity (p < 0.001). However, the combination of BRCA1 siRNA with E6 or with E7 caused little or no additional increase in hTERT activity beyond that observed in the presence of control siRNA. The ability of the BRCA1 siRNA to knock down BRCA1 protein levels is illustrated in Fig. 11B. The potential significance of these findings is considered under the “Discussion.”

DISCUSSION

We showed that the HPV oncoproteins encoded by the “early genes” E7 and E6 interact with the tumor suppressor protein BRCA1 and alter its activity. Thus, both E7 and E6 completely rescue the BRCA1-mediated repression of ER-α transcriptional activity, and each oncoprotein interacts with BRCA1 in vitro and associates with BRCA1 in vivo. Previously, we showed that BRCA1 inhibits telomerase activity, in part, through inhibition of TERT promoter activity and repression of c-Myc-mediated transcription via the E-box DNA element (29). The presence of BRCA1 on the TERT promoter suggests a direct mechanism of repression (29). Here we showed that co-expression of E6 or E7 with wtBRCA1 partially reverses the wtBRCA1-mediated inhibition of hTERT and E-box luciferase reporters. In the absence of exogenous wtBRCA1, E6 and E7 stimulated the hTERT and E-box-Luc activity by about 2.5–4-fold in different experiments. Thus, we cannot rule out the possibility that some of the “rescue” activity of E6 and E7 is because of a BRCA1-independent effect on the hTERT and E-box promoters. This does not appear to true for the E6 and E7 rescue in ER-α activity, because in the absence of wtBRCA1, E6 and E7 caused very modest changes in ER-α activity.

The ability of E6 to stimulate hTERT activity is supported by the prior finding that E6 stimulates hTERT promoter and telomerase activity in cooperation with c-Myc (33). However, it is possible that E6 and E7 could stimulate hTERT activity in part by antagonism of the endogenous BRCA1. The finding that knockdown of endogenous BRCA1 by RNA interference does not enhance E6- or E7-stimulated hTERT activity suggests that the oncoproteins are acting in part through BRCA1. Furthermore, the finding that in the presence of wtBRCA1, the fold increase in hTERT and E-box-Luc activity caused by E6 or E7 was considerably greater than in its absence suggests that E6 and E7 function in part to antagonize the activity of BRCA1. Point mutations of the zinc finger domains, which disrupt the interaction with BRCA1, abrogated the ability of E6 and E7 to stimulate hTERT or E-box-Luc activity and severely attenuated their ability to rescue the wtBRCA1 inhibition of
hTERT, E-box, or ER-α activity. A caution here is that findings obtained using human breast cancer cells cannot necessarily be extrapolated to cervical cancer cells or precursor epithelium.

Previous studies have revealed interactions between BRCA1 and two other tumor suppressor proteins, RB1 and p53 (34–39). Thus, BRCA1 physically binds to RB1 (through sites in the N- and C termini of BRCA1) and is required for the RB1-mediated G1 cell cycle checkpoint (34–36). Furthermore, BRCA1 binds to p53 (again, through several different sites on BRCA1) and acts as a co-activator of p53-mediated transcription (37–39). Whether the interaction of E6 or E7 with BRCA1 alters the ability of BRCA1 to potentiate the activities of p53 or RB1 is also unclear at the present time. Our studies have not formally addressed the ability of BRCA1 to modulate the transforming activities of the E6 and E7 proteins. However, looked at from another point of view, our E-box-Luc assays suggest that exogenous BRCA1 can antagonize the binding of E6 and E7 to stimulate E-box-Luc activity, although the antagonism is relatively weak. In this regard, it has been documented previously (40) that BRCA1 inhibits c-Myc-mediated cellular transformation.

We performed a high resolution analysis of the BRCA1-ER-α structural interaction to better delineate the mechanism(s) by which BRCA1 inhibits ER-α activity (24). This study revealed two interaction sites for ER-α on BRCA1, one within amino acids 67–100 and another (and weaker) site within amino acids 101–133. We identified a site within BRCA1 amino acids 86–95 with similarity to an extended helix-type co-repressor motif and an human immunodeficiency virus nuclear export signal that is essential for interaction with and repression of ER-α (24). Most interestingly, the present study showed that BRCA1 amino acids 67–100 participate in the binding to E6 and E7, although another site(s) in the C terminus of BRCA1 (amino acids 1532–1749) also participates in binding. The C-terminal site includes one of the two BRCA1 C-terminal repeats (BRCT1, amino acids 1650–1738). BRCT2 spans amino acids 1764–1854 (41). BRCT-like repeats are found in a number of proteins involved in DNA repair and DNA damage-responsive cell cycle checkpoints (42). The BRCT repeats of BRCA1 interact with a variety of proteins involved in transcriptional regulation and/or DNA repair, including C-terminal interacting protein, histone deacetylases, RB1, and RB1-associated proteins (RbAp46 and RbAp48), RNA helicase A, phosphopeptides that function in DNA damage signaling, and BACH1 (a protein involved in repair of double-stranded DNA breaks) (see Ref. 42 and reviewed in Ref. 43). Whether the interaction of BRCA1 with E6 or E7 disrupts these interactions or their functional consequences remains to be determined. Because

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**FIGURE 10.** Ability of zinc finger-defective mutant E6 and E7 oncoproteins to rescue the wtBRCA1-mediated repression of the hTERT-Luc (A) and E-box-Luc (B) promoters. Assays were performed as described in Fig. 8 legend. Luciferase activity was expressed as a percentage of the positive control (reporter only, no wtBRCA1, E6, or E7). The values plotted are means ± S.E. of four replicate wells.

**FIGURE 11.** Effect of knockdown of BRCA1 on the ability of the E6 and E7 oncoproteins to stimulate hTERT promoter activity. A, hTERT promoter assays. Subconfluent proliferating MCF-7 cells in 24-well dishes were preincubated for 48 h with a previously validated BRCA1 siRNA (29) or the control siRNA (50 nM); transfected overnight with wtE6, wtE7, or pcDNA3 vector; and post-incubated for 24 h to allow gene expression. Luciferase activity was expressed as a percentage of the positive control (hTERT-Luc only, no siRNA, E6, or E7). The values shown are means ± S.E. of four replicate wells. The data are shown on a linear scale to more easily delineate 2–3-fold changes in reporter gene activity. B, effect of siRNAs on BRCA1 protein levels. Subconfluent proliferating MCF-7 cells were treated with no siRNA (vehicle only), BRCA1 siRNA, or control siRNA (50 nM) for 72 h and harvested for Western blotting to detect BRCA1 or α-actin (control for loading and transfer).
BRCA1 Interaction with HPV

BRCA1 is a component of the RNA polymerase II holoenzyme, the interaction of E6 and E7 with BRCA1 could reflect a mechanism through which these HPV oncoproteins subvert the cellular transcriptional machinery, but this is conjectural at present.

These studies along with the findings that BRCA1 mutation carriers have an increased risk for cervical cancer and that a subset of sporadic cervical cancers exhibit hypermethylation of the BRCA1 promoter (see Introduction) raise the possibility that BRCA1 plays a role in the molecular pathogenesis of cervical cancer. However, this hypothesis remains to be proven. We note here that findings obtained in other cell types (e.g. breast cancer cells) cannot necessarily be extrapolated to cervical epithelial cells. Cervical cancer is one of the common cancers occurring in women worldwide (44, 45). Infection with an oncogenic HPV subtype (e.g. type 16 or 18) appears to be an obligate event in the pathogenesis of cervical cancer that is demonstrable in nearly all cancer cases. In fact, HPV vaccines are currently undergoing preclinical and clinical testing as a means of prevention of cervical cancer (46). Among the different components of the HPV genome, the most commonly expressed viral proteins and the ones that are most closely associated with cervical cancer development are the E6 and E7 oncoproteins (47). These oncoproteins are thought to cause cellular transformation, in part, by causing the degradation or functional inactivation of p53 (E6) and RB family proteins (E7) and by causing immortalization via expression of telomerase activity (E6) (47). E6 cooperates with c-Myc, a transactivator, to activate the telomerase reverse transcriptase (hTERT) promoter (33, 48). Several functions of BRCA1 are potentially relevant to the development of cervical cancer, including the ability of BRCA1 to inhibit ER-α signaling (11–14). Thus, there is evidence to suggest a role for estrogen in stimulating cell growth during the pathogenesis of cervical cancer in animal models and in humans (49–51), although the hormonal etiology of cervical cancer is not as well established, as it is in the case of breast and endometrial cancers. In this regard, the "cervical transformation zone" (squamo-columnar junction), where cervical cancers typically originate, appears to be particularly sensitive to estrogen-induced cellular proliferation (51).

Although most BRCA1 mutant breast cancers are ER-α negative (52), the ability of BRCA1 to inhibit ER-α activity may contribute significantly to the development of BRCA1-mutant breast cancers based on a series of findings indicating that hormonal factors contribute to breast cancer risk in BRCA1 mutation carriers and that hormonal prophylaxis (bilateral oophorectomy) significantly reduces the incidence of BRCA1 mutant breast cancers (53–55). In addition, studies of a mouse transgenic model featuring a mammary-targeted deletion of the full-length Brca1 isoform revealed that tamoxifen, an anti-estrogen with mixed antagonist/agonist activity, significantly increased the incidence of mammary cancer (56). Correlating with this finding, knockdown of endogenous BRCA1 in cultured MCF-7 cells caused an increase in the agonist activity of tamoxifen (56). Most interestingly, HPV DNA sequences have been detected in human breast tumor tissue and areolar samples (57), but a pathogenetic role for HPV's in human breast carcinogenesis has not been established.

Finally, based on our findings, it appears likely that the mechanism(s) by which E6 and E7 inactivate BRCA1 is not identical to those pertaining to p53 or RB1 for the following reasons. 1) BRCA1 is not degraded in response to E6 and E7, as are p53 and RB1, respectively. 2) Amino acids 19–38 of E7 (which contain the RB1-binding motif LXCXE, see Fig. 1A) do not bind BRCA1, whereas amino acids 38–98 (which are not required for RB1 binding) mediate the binding to BRCA1. 3) Both E6 and E7 can inactivate BRCA1, although E6 selectively targets p53 and E7 selectively targets RB1. The precise interacting site for p53 on E6 is unclear, although proteolysis of p53 is dependent upon the zinc finger structures, particularly the second zinc finger (58). At present, it is unclear if there is overlap between the binding sites for p53 and BRCA1 on E6.

In conclusion, we have demonstrated and partially mapped a physical interaction between BRCA1 and oncogenic HPV-E6 and E7 proteins that results in the antagonism of several different functional activities of BRCA1. It remains to be discovered whether BRCA1 plays a role in the initiation of cervical cancer, but based on these findings, further studies are warranted.

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