

Novel Interaction between HPV E6 and BARD1 (BRCA1-Associated Ring Domain 1) and Its Biologic Roles

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Human papillomaviruses (HPVs), which are associated with the majority of cervical cancers, encode a transforming protein, E6, which interacts with the p53 tumor suppressor protein. There is a wide effort focused on searching for the target of the involvement of p53-independent HPV 16 E6-interacting proteins. We identified Breast Cancer 1 Gene (BRCA1)-associated ring domain protein 1 (BARD1) as a binding partner of E6 and investigated its biological function in cervical cancer cells. *In vivo* co-immunoprecipitation assay was performed to determine whether E6–BARD1 interaction occurred. We then used a degradation assay to determine whether E6-mediated inactivation of BARD1 transactivation function was associated with BARD1 degradation. A mutation assay revealed the site of interaction of E6 with BARD1. The effect of BARD1 on p53 transcriptional activity was tested using BARD1 knockdown and overexpression systems. BARD1 was not degraded by E6, and, instead, formed a physical complex with E6. Moreover, the mutations of the metal motif zinc-finger region decreased the ability of E6 to interact with BARD1. Transient transfection of BARD1 increased the p53-mediated activation of p21^{WAF1} promoter despite the presence of E6. Additionally, the existence of BARD1 inactivated the expression of E6 in cervical cancer cells. These findings suggest that BARD1 may regulate the transcriptional activities of p53 as tumor suppressors.

Introduction

HUMAN PAPILLOMAVIRUSES (HPVs) ARE DNA VIRUSES that induce the transformation of epithelial cells during the course of infection. Infection with specific types of HPV appears to be an essential step in the development of invasive cervical cancer and/or its precursory lesions, for example, squamous intraepithelial lesions (Hansen and Oren, 1997). HPV E6 and E7 oncogenes bind to inactivate critical tumor suppressor proteins, which enables the virus to override checkpoints that regulate cell proliferation (Dyson *et al.*, 1989; Scheffner *et al.*, 1990).

The HPV E6 protein consists of 158 amino acid residues and contains 2 zinc-finger-binding motifs (Kanda *et al.*, 1991; Lipari *et al.*, 2001). This protein is thought to promote cellular proliferation by stimulating the degradation of the p53 tumor suppressor protein via formation of a trimeric complex composed of E6, p53, and the cellular ubiquitination enzyme, E6-AP (Huibregtse *et al.*, 1993; Pim *et al.*, 1994). E6-stimulated degradation interferes with such biological functions of p53 and thus perturbs the control of cell cycle progression, which finally leads to increased tumor cell growth (Hansen and Oren, 1997). Various cellular proteins have been reported to interact with the high-risk type HPV E6 proteins (e.g., Bak,

c-Myc, E6-BP/ERC 55, E6TPI, CBP/p300, ORF-3, Mcm 7, Paxillin, hD1g, MAGI-1, MUPP-1, and hScrib) and to be targeted for degradation in an E6-AP-dependent (Bak, c-Myc, Mcm 7, and hScrib) or E6-AP-independent (hD1g, MAGI-1, and MUPP-1) manner (Patel *et al.*, 1999; Mantovani and Banks, 2001). HPV E7 is the major transforming oncogene of HPV that binds cellular proteins of the pRb tumor suppressor family. The binding of E7 to the active form of pRb leads to the release of E2F transcription factors, which then stimulates entry into the S-phase of the cell cycle and leads to cell replication (Boyer *et al.*, 1996).

Here, we performed yeast two-hybrid screening to search for HPV 16 E6 interacting proteins, and found that BRCA1-associated ring domain protein 1 (BARD1). BARD1 is described as a nuclear protein that is associated with the breast cancer susceptibility gene product, BRCA1 (Wu *et al.*, 1996). The major link between BARD1 and breast/ovarian cancer reflects a strong, stable interaction between BARD1 and BRCA1, which colocalize in the nucleus (Jin *et al.*, 1997) and form complexes that can be purified from living cells (Chiba and Parvin, 2001). BARD1 has been labeled a putative tumor suppressor because its gene has been observed to be mutated in a subset of breast, ovarian, and uterine cancers. BARD1 is structurally related to BRCA1 in that it harbors an

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amino-terminal RING domain and two carboxy-terminal BRCA1 (BRCT) motifs (Wu *et al.*, 1996; Jang and Lee, 2004). *In vivo* BRCA1 exists as a heterodimer with BARD1, a protein that displays a similar configuration of RING and BRCT motifs. Colocalization of BARD1 with BRCA1 and other repair proteins indicates that it plays a role in DNA repair. A potential role of BARD1–BRCA1 complexes in the ubiquitination of RNA pol II and the interaction of BARD1 with polyadenylation factor, CstF-50, which inhibits mRNA processing, provides mechanisms for tumor suppression (Kleiman and Manley, 2001). A BRCA1-independent function of BARD1 is its interaction with NF- κ B and the modulation of its transcriptional activity (Dechend *et al.*, 1999).

BARD1-repressed cells show a prolonged S-phase, suggesting a role of BARD1 in normal proliferation; the cells also exhibit genetic instability, loss of growth inhibition by contact, and loss of morphogenetic properties (Irminger-Finger *et al.*, 1998). The increase in the expression of BARD1 in apoptotic tumor cells is suggestive of its function in a novel tumor suppressor pathway, and is consistent with the recently described role of BARD1 in apoptotic signaling (Irminger-Finger *et al.*, 2001). Cell death *in vivo* and *in vitro* is accompanied by increased levels of BARD1 protein and mRNA, while BARD1-repressed cells are defective for the apoptotic stress response. The proapoptotic activity of BARD1 involves its binding to and stabilizing p53, but this activity is independent of BRCA1. Loss of BARD1 function is associated with carcinogenesis, and exogenous BARD1 expression may lead to repressed tumor growth.

We examined the sites of interaction of E6 and BARD1, the biological significance of this interaction, and the role of BARD1 in p53-mediated transcriptional activation. Taken together, these observations suggest that BARD1 may act as a p53 tumor co-suppressor and as a transcriptional regulator of E6 in cervical carcinogenesis.

Materials and Methods

Cell culture

CaSki and Cos-1 cells used in our experiments were maintained as monolayers in Dulbecco's modified Eagle's medium (DMEM) with 10% (v/v) fetal bovine serum (Gibco BRL, Gaithersburg, MD) and incubated at 37°C in a humidified atmosphere of a 5% (v/v) carbon dioxide in air.

cDNA construction

All cDNAs were made according to standard methods (Sambrook *et al.*, 1989) and were verified by sequencing. The multicopy yeast expression plasmids used in the two-hybrid assays were pBTM116 (a Trp1-LexA DBD vector) and pASV3 (a Leu2-VP16 acidic transactivation domain vector). In these plasmids, the target fusion genes are expressed under control of the ADH1 (alcohol dehydrogenase 1) and phosphoglycerate kinase promoters, respectively (Hofmann *et al.*, 2000). Deletion and point mutants of the E6 genes were created by PCR amplification and subcloned into pBTM116. Mutated HPV 16 E6 genes Δ 9–13, Δ 101–105, Δ 111–115, Δ 138–147, 45Y/47Y/49H, R39G, L60G, D120G, and K115E have been described elsewhere (Crook *et al.*, 1991). The E6 genes were subcloned into the *Hind* III site of pBS (+) and designated pBS-16E6 66G, -136G, and -66G/136G. HPV 11 E6, 18 E6, and pBTM116 were used (Crook *et al.*, 1991; Nakagawa *et al.*, 1995).

Yeast two-hybrid assays

A human HeLa cDNA library (BD Biosciences, Palo Alto, CA) in the prey plasmid, pGAD10 (BD Biosciences), was screened for proteins that interact with E6 using the yeast reporter strain, L40 [MATa, his3 Δ 200, trp1-901, leu2-3, 112, ade2, LYS::(*lexAop*)₄-HIS3, and URA3::(*lexAop*)₈-lacZ] (Park *et al.*, 2000). The prey and bait plasmids were cotransformed into L40 using lithium acetate. Transformed cells were spread directly on minimal medium lacking histidine, leucine, and tryptophan, and supplemented with 5 mM 3-aminotriazole. Positive clones were isolated and retested for β -galactosidase (β -gal) activity on permeable cells. Library plasmids from positive isolates were transformed into and recovered from *Escherichia coli* strain HB101 (leu2⁻); these isolates were then analyzed by restriction digests. Unique inserts were sequenced and analyzed by comparison to the GenBank™ sequence data bank. The longest insert was systematically tested for interactions with a protein containing the LexA DBD fused to E6 mutants by β -gal assays (Park *et al.*, 2001). To precisely map the BARD1 interaction domain on E6, full-length and deletion derivatives of E6 were fused with a LexA DBD by subcloning into vector pBTM116. The resulting VP16 AD-BARD1 fusion vector, pASV3, was cotransformed with a pBTM116 derivative encoding the LexA DBD-E6 fusion protein.

Yeast transformation and β -gal assay

The L40 yeast reporter strain was used to monitor transactivation of the E6 mutant cloned in a pBTM116 vector. Mutants in pBTM116 were introduced into L40 using the lithium acetate procedure. Transformed cells were spread directly on minimal medium lacking tryptophan. Positive clones were isolated, lysed by three freezing–thawing cycles, and then tested for β -gal activity according to a standard procedure (Qin *et al.*, 1999), except that reactions were performed in 96-well plates.

Co-immunoprecipitation assay

Cos-1 cells cultured in six-well plates were cotransfected with 1 μ g of pcDNA3-HA-BARD1 and pcDNA3-2X Flag-HPV 16 E6. The cells were washed in phosphate-buffered saline (PBS), and cell lysates were prepared by the addition of 1 mL of ice-cold Radio-Immunoprecipitation Assay (RIPA) buffer [250 mM NaCl, 50 mM HEPES (pH 7.0), 0.1% NP-40, and 5 mM ethylenediaminetetraacetic acid (EDTA)] supplemented with protease inhibitors and precleared by incubation with protein A/G-agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h. The precleared lysates were then immunoprecipitated with mouse anti-HA monoclonal antibody (mAb, 1:200 dilution; Sigma, St. Louis, MO) and mouse anti-flag M2 monoclonal antibody (mAb, 1:200 dilution; Sigma), and protein A/G-agarose beads overnight at 4°C. The beads were washed five times in RIPA buffer and twice with PBS, and the immune complexes were released from the beads by boiling in sample buffer for 5 min. Following 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), immunoprecipitation products were analyzed by immunoblotting.

Immunoblot

For immunoblotting, the cells were lysed in RIPA buffer on ice for 1 h. The insoluble materials were removed by centri-

fugation at 12,000 rpm for 20 min at 4°C. The supernatant was then subjected to SDS-PAGE and immunoblotting. The blots were blocked in PBS containing 5% skim milk and 0.05% Tween 20, and were incubated with primary and horseradish peroxidase-conjugated secondary antibodies. For all of the protein detection procedures, BARD1 (Santa Cruz Biotechnology), p53 (Novocastra Lab., Newcastle, UK), and β -actin (Sigma) were used as primary antibodies at 1:200 dilution. Antibody binding was determined using an ECL kit (Amersham Bioscience, Arlington Heights, IL).

Transient transfection, CAT ELISA, and luciferase assay

p53-null Cos-1 cells were transiently transfected using the Lipofectamine plus reagent (Invitrogen, Carlsbad, CA) according to the instructions of the manufacturer. In six-well plates, total amounts (2 μ g) of DNAs were kept constant by the addition of SV40-driven β -gal internal control plasmid and the respective test plasmids as indicated in the text. After overnight transfection, cells were washed, fed with the complete medium, and further incubated for 24 h. Cells were then collected and extracted. Extracts were cleared by centrifugation, and protein concentrations were determined with the Bio-Rad protein assay dye reagent (Bio-Rad, Philadelphia, PA). β -gal activity was determined in 96-well plates. A 30–70 μ L aliquot of the clear lysates was tested for chloramphenicol acetyltransferase (CAT) concentrations in the CAT enzyme-linked immunosorbent assay (ELISA), performed according to the instructions of the manufacturer (Roche Molecular Biochemicals, Mannheim, Germany). The CAT concentration of each sample was normalized with respect to β -gal activity.

For the luciferase assay, cells were seeded in a 12-well culture plate at a density of 1.5×10^5 cells/well. Transfection was performed using Lipofectamine plus reagent (Invitrogen) with Gal4-tk-luciferase, pG4M-poly II-BARD1, 2X Flag-HPV 16 E6, and SV 40-driven β -gal expression vector as an internal control. After transfection for 4 h, cells were washed, fed with 5% charcoal-stripped medium, and incubated for an additional 20 h. Cells were then washed with ice-cold PBS, collected, resuspended in 100 μ L of luciferase lysis buffer (Promega, Madison, WI), and subjected to three freeze-thaw cycles. Cell lysates were cleared by centrifugation (13,000 rpm, 10 min, 4°C), protein concentrations were determined using a Bio-Rad protein assay dye reagent, and luciferase activity was measured by adding 20 μ L luciferin (Promega) into 30 μ L of lysates using an Analytical Luminescence luminometer according to the instructions of the manufacturer. To measure β -gal, 10 μ L of cell lysate was incubated in 500 μ L of LacZ buffer (50 mM sodium phosphate, 10 mM potassium chloride, 1 mM magnesium sulfate, and 0.005% β -mercaptoethanol) with 100 μ L of 4 mg/mL *p*-nitrophenyl β -D-galactopyranoside (Amersham Bioscience) at 37°C. β -gal activity was determined in 96-well plates that were read at 405 nm using ELISA reader. The luciferase activities were normalized to the β -gal activity expressed from the cotransfected β -gal expression vector in relative luciferase units.

Preparation of siRNA and transfection

Synthetic siRNAs against mRNAs encoding BARD1 were obtained from Invitrogen. The respective target sequence was for BARD1 5'-AAAGGCTTCTGCAAGATCTG-3' (nucleo-

tides 660–680). The selected siRNAs were BLAST-searched against the human genome sequence to ensure only that one gene was targeted, whereas the control (nonsilencing) siRNA used had no known overlap. The siRNAs ("Stealth RNA") were chemically synthesized and annealed with >97% purity (Invitrogen). For the introduction of siRNA in the six-well plates, we transiently transfected it to CaSki cells with 4 μ L Lipofectamine 2000 (Invitrogen) and siRNA. Cells were assayed for silencing 48–96 h after transfection.

Preparation of BARD1 expression vector and transfection

The BARD1 expression clone was produced by cloning the coding sequence of the human BARD1 cDNA into the *Kpn* I and *Not* I sites of pcDNA3-HA. Transfections were performed in a reproducible manner using Lipofectamine (Invitrogen). About 1–4 μ g of DNA was transfected for each 100 mm dish.

RT-PCR

Total RNA was prepared from cell cultures with the RNeasy[®] Mini kit (Qiagen, Hilden, Germany). The primers used in this study were as follows: HPV 16 E6 (forward: 5'-AAGGCGTAACCGAAATCGG-3' and reverse: 5'-CATATACCTCAGTCGCAG-3') and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (forward: 5'-CCATGTTTCGTCATGGGTGTGAACCA-3' and reverse: 5'-GCCAGTAGA GGCAGGGATGATGTTTC-3'). mRNA levels of GAPDH were used to normalize the amount of sample RNA.

Statistical analysis

All observations were confirmed by at least three independent experiments. The Student's *t*-test was used to evaluate the statistical significance of mean values. Values of $p < 0.05$ were considered to be significant and are indicated by asterisks in the figures.

Results

E6 protein interacts with BARD1

The yeast two-hybrid system was used to identify proteins that interacted with HPV 16 E6. A total of 52 unique library plasmids were obtained from the initial screen. DNA sequencing and subsequent GenBank searches indicated that five of these clones contained partial sequences of BARD1. Figure 1A shows a schematic diagram of the BARD1 cDNA.

To determine whether HPV 16 E6–BARD1 interaction occurred, we performed an *in vivo* co-immunoprecipitation assay after transfection with the 2X-flag-tagged HPV 16 E6 and the HA-tagged BARD1 in Cos-1 cells. Using immunoblotting, the immunoprecipitates were then for the presence of HPV 16 E6 and BARD1 interaction, and the results obtained are shown in Figure 1B. These data indicate that HPV 16 E6 and BARD1 combine to form a physical complex *in vivo*.

BARD1, unlike p53, is not susceptible to E6-mediated degradation

Because p53 is inactivated by E6-dependent degradation, we then attempted to determine whether E6-mediated inactivation of BARD1 transactivation function is correlated with

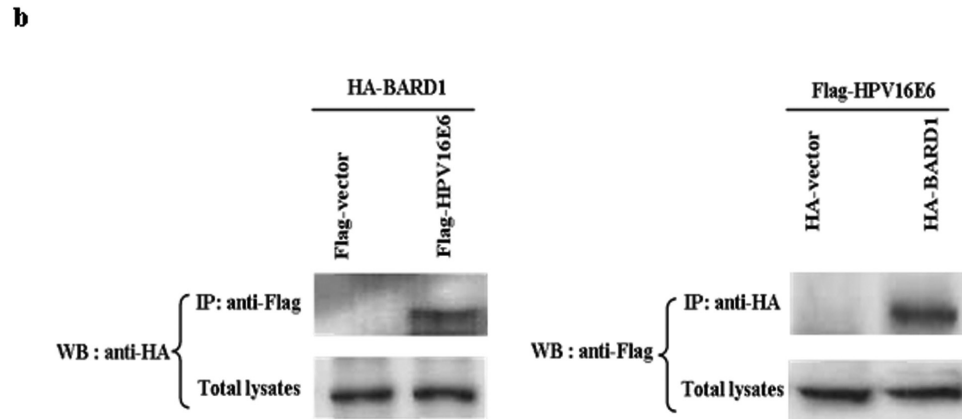
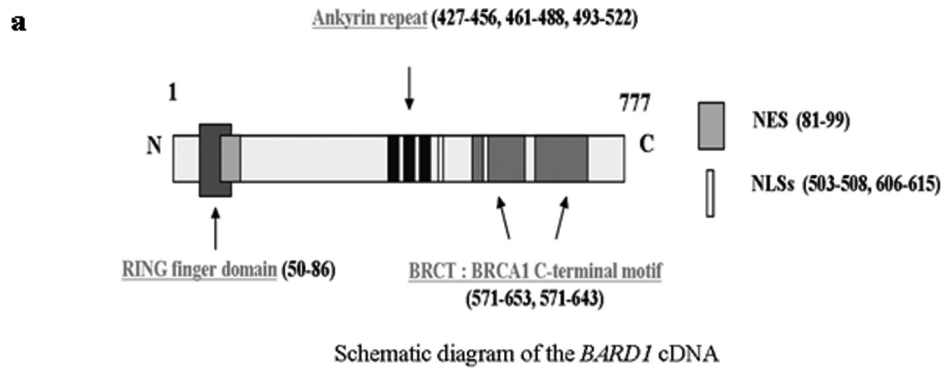


FIG. 1. HPV E6 protein interacts with BARD1. (A) Identification of BARD1 by yeast two-hybrid screening using a HeLa cDNA library. Schematic diagram of the *BARD1* cDNA. The RING-finger domain, ankyrin repeats, and BRCT sequences are shown. (B) *In vivo* interaction assay by co-immunoprecipitation. After transfection with HA-tagged BARD1 and 2X-flag-tagged HPV 16 E6 protein in Cos-1 cells, cells were lysed in RIPA buffer supplemented with protease inhibitors and precleared by incubation with protein A/G agarose for 1 h. Immunoprecipitation was carried out with anti-HA and anti-flag M2 antibody. After resolution by 10% SDS-PAGE, immunoblot was carried out.

BARD1 degradation. As expected, p53 was degraded by the introduction of HPV 16 E6; however, BARD1 was resistant to the E6-mediated degradation (Fig. 2).

BARD1 raises the concentration of available p53 for reporter activation by inhibition of E6

We tested whether BARD1 might have an effect on endogenous wild-type p53 in CaSki (HPV 16 E6-positive) cervical carcinoma cells. These cells express wild-type p53 protein, but p53 levels are low due to the presence of the viral HPV E6 protein, which targets p53 for degradation. We transfected CaSki cells with the same amount of p53-dependent p21-CAT reporter construct and treated the cells with p53 and/or BARD1. The transcriptional level was assessed by measuring β -gal activity from cell extracts. A slight increase in reporter gene activation was observed (Fig. 3A). Because of the low p53 levels, the transfection with BARD1 did not have a significant effect on the p53-mediated transcriptional activation. However, it seemed that BARD1 led to a stimulation of p53 transcriptional activation. Cotransfection with a fixed amount of wild-type p53, E6, and increasing amounts of BARD1 (0, 1, 2, and 3 μ g) in Cos-1 cells induced the restoration of p53 transcriptional activation (Fig. 3B). The addition of increasing amounts of BARD1 raises the level of p53-mediated reporter gene activation. The transcriptional activation of BARD1 was

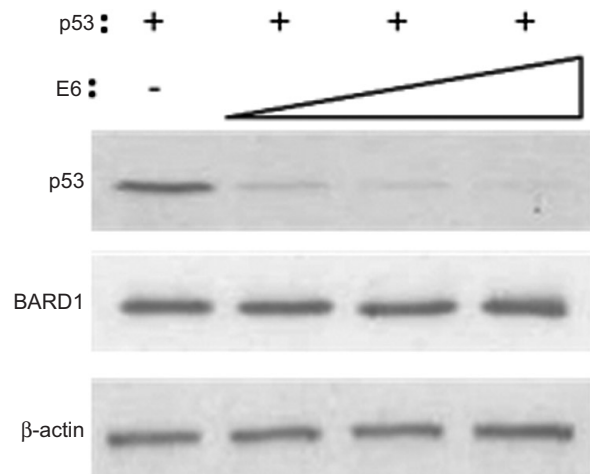


FIG. 2. BARD1 is not susceptible to E6-mediated degradation. BARD1 is not susceptible to HPV E6-mediated degradation, whereas p53 is inactivated by E6-dependent degradation. After transfection with HA-tagged p53 and 2X-flag-tagged HPV 16 E6 in Cos-1 cells, cells were lysed with RIPA buffer. Immunoblot was carried out with anti-HA antibody. p53 was degraded by HPV 16 E6. Conversely, after transfection with HA-tagged BARD1 and 2X-flag-tagged HPV 16 E6 in Cos-1 cells, immunoblot was carried out with anti-HA antibody. BARD1 was not degraded by HPV 16 E6.

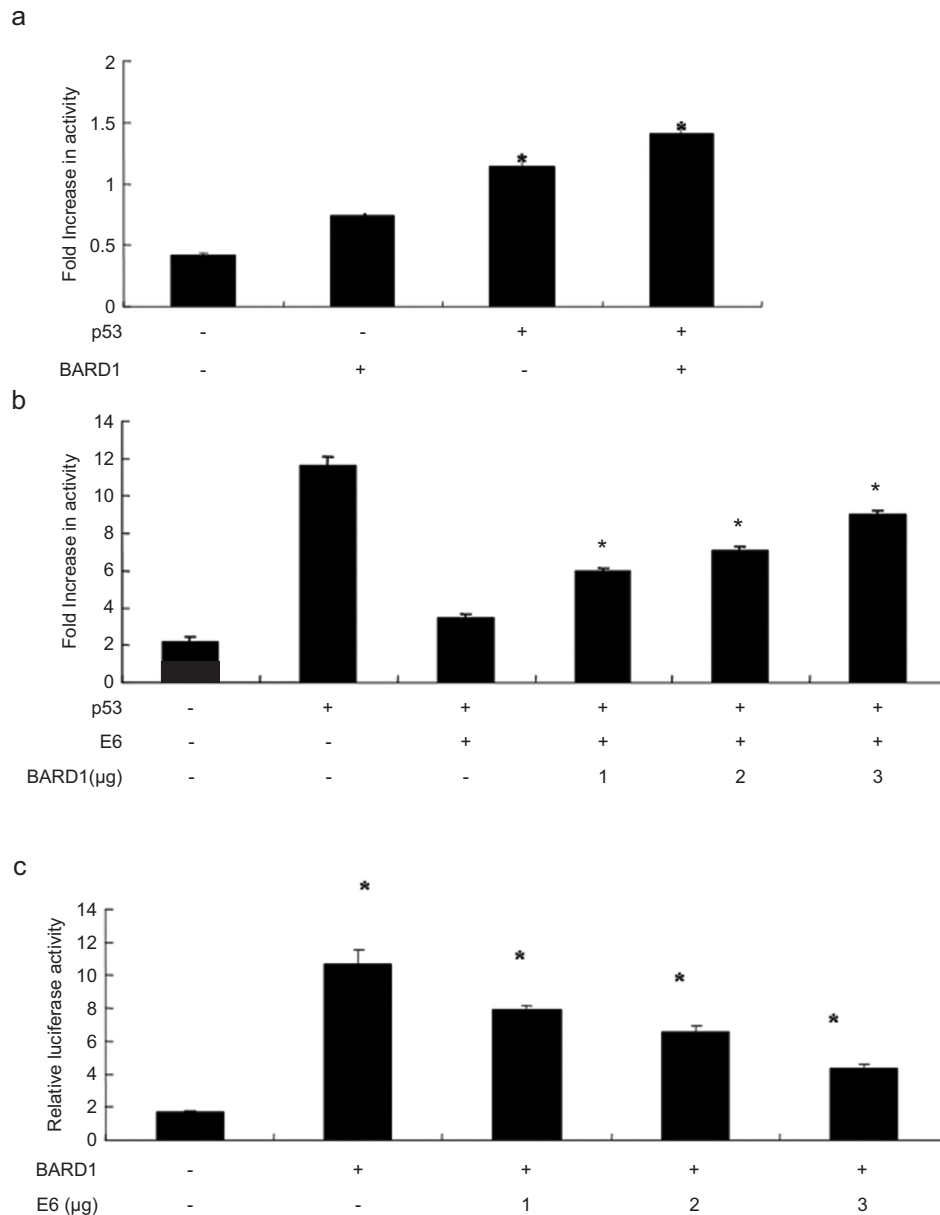


FIG. 3. BARD1 activates the transcriptional activity of p53. (A) An increase of p53-mediated transcriptional activation by transfection of exogenous BARD-1 in CaSki cells. These experiments were performed in triplicate ($*p < 0.05$). (B) Dose-dependent increase of p53 transcriptional activation by transfection with a fixed amount of wild-type p53, HPV 16 E6, and increasing amounts of BARD1 in Cos-1 cells. Cos-1 cells were transfected with a combination of p53 and BARD1 together with p21^{WAF1} promoter-CAT reporter plasmid. Data represent the mean of at least three independent transfections. Fold stimulation was calculated by dividing the amount of CAT enzyme of treated cells, as determined by CAT ELISA, by that of untreated control cells. These experiments were performed in triplicate ($*p < 0.05$). (C) HPV E6 proteins repress the transcriptional activity of BARD1 in Cos-1 cells. Relative luciferase activity was determined in triplicate, and data were normalized to β -gal activity. These experiments were performed in triplicate ($*p < 0.05$).

assessed by luciferase assay after transfection with pG4M-poly II BARD1 and HPV 16 E6 in Cos-1 cells. The level of transcriptional activation of BARD1 was gradually reduced in an HPV 16 E6 dose-dependent manner (Fig. 3C).

Taken together, these results suggest that BARD1 inhibits E6 and thus raises the concentration of available p53 for activation of the reporter.

Amino acid residues of E6 are responsible for the interaction of BARD1 function

To determine the amino acid residues involved in the inactivation of BARD1 function, we used E6 mutants (Crook *et al.*, 1991; Nakagawa *et al.*, 1995) fused to the LexA DBD in the pBTM116 vector and cotransformed these constructs into

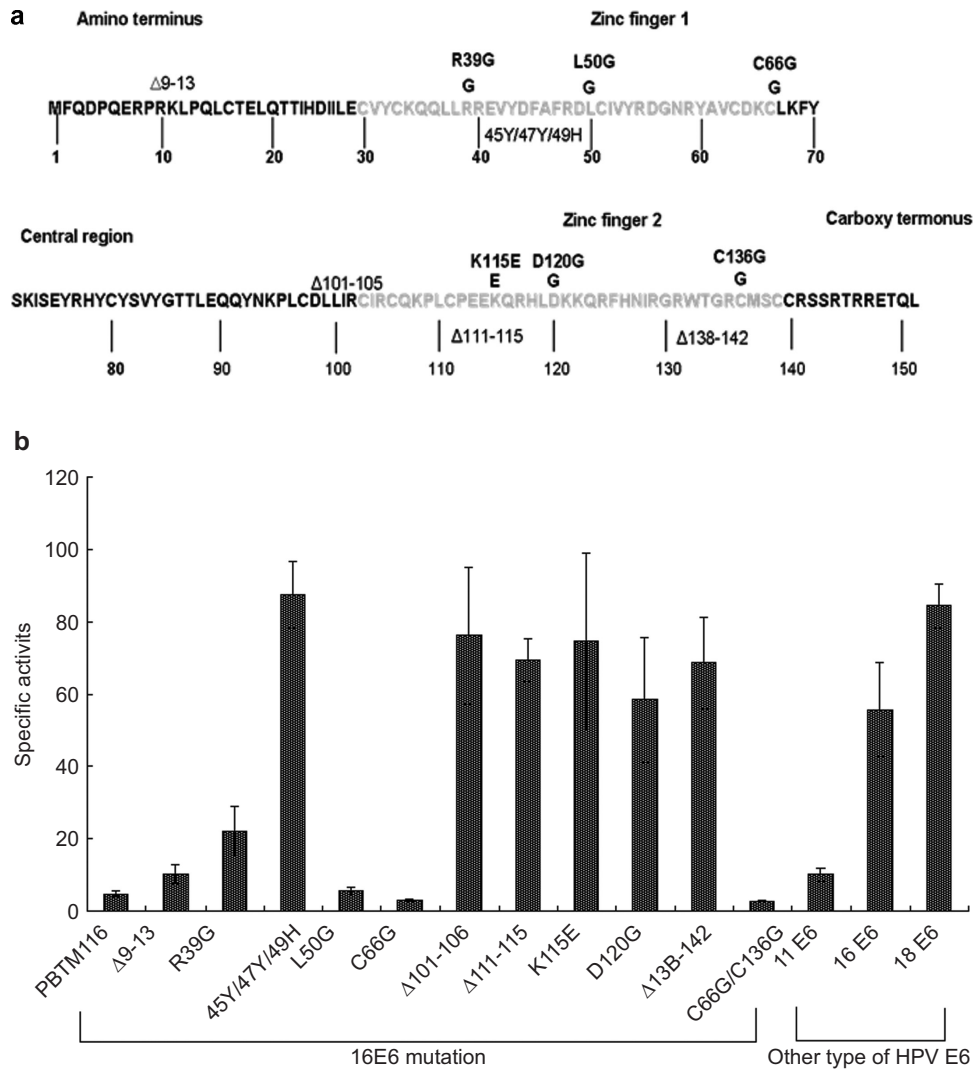


FIG. 4. Amino acid residues of E6 responsible for the interaction of BARD1 function. (A) Amino acid sequence of HPV 16 E6 showing various mutations. The shadow regions indicate zinc-finger motifs. (B) Mapping of the specific region of HPV 16 E6 responsible for interaction with BARD1. HPV 16 E6 45Y/47Y/49H, 16E6 Δ 9-13, Δ 101-105, Δ 111-115, and Δ 138-142, R39G, L50G, C66G, D120G, K115E, C136G, and C66G/C136G were used. Yeast two-hybrid and β -gal assays were performed using LexA-fused HPV 16 E6 mutants and other types of HPV E6 (in pBTM116).

the L40 yeast strain. BARD1 transactivation was measured by β -gal activity. The G substitutions of C66 and C66/C136 drastically reduced the ability of E6 to inhibit BARD1 transcriptional activity. Because the E6 protein contains two zinc-fingers composed of four metal-binding motifs (C-X-X-C), the disruption of the zinc-fingers by the substitution of C with G may result in destabilization of the E6 structure. Additionally, the five-amino-acid deletion mutant within the amino-terminal portion Δ 9-13 and the G substitution mutants of R39 and L50 (R39G and L50G) within zinc-finger 1 could inactivate the transactivation function of BARD1. Three mutants with deletions and substitution within zinc-finger 2, Δ 111-115, D120G, and K115E, two mutants with partial deletions within zinc-finger 2, Δ 101-105 and Δ 138-142, and 45Y/47Y/49H with triple substitution within zinc-finger 1 still retained interaction with BARD1. Notably, only the interaction of BARD1 by high-risk E6 was found (Fig. 4). Taken together, these results suggest that zinc-finger 1 of E6 is more important for BARD1 interac-

tion than other regions of E6. When compared with the levels of E6-mediated inactivation between p53 and BARD1, the levels of E6 wt-mediated inactivation were set to 100%. Other E6 mutant-mediated percentages of inactivation were represented by the relative ratio. The results are summarized in Table 1.

The repression of BARD1 induces the reduction of p53-mediated transcriptional activation

We suggested that BARD1 increases p53-mediated transcriptional activation. Conversely, to investigate the role of the repression of BARD1 on p53 transcriptional activation, we reduced the level of BARD1 using an RNA interference system in CaSki cells. BARD1 mRNA and protein expression by siRNA were measured by immunoblot using a polyclonal antibody and RT-PCR using a specific primer (Fig. 5A).

We transfected CaSki cells with the same amount of p53-dependent p21-CAT reporter construct, and we then treated

TABLE 1. COMPARISON OF HPV-16 E6 MUTANT ACTIVITY IN THE INACTIVATION OF P53 AND BARD1

HPV 16-E6 mutant	p53	BARD1
	Inactivation (%) ^a	Inactivation (%)
E6-wt	100	100
L50G	40.1	10.2
R39G	101.2	39.8
C66G	30.1	5.5
K115E	91.1	134.0
D120G	102.5	104.9
C66G + C136G	7.1	5.0

^a Values described by Park *et al.* (2001).

the cells with p53 and/or BARD1 siRNA. CaSki cells express wild-type p53 protein, but p53 levels are very low because of the presence of viral HPV E6 protein, which targets p53 for degradation. The presence of the viral E6 protein causes the repression of wild-type p53 in CaSki cells. Furthermore, the repression of p53-stabilizing BARD1 by siRNA further decreases

p53 transcriptional activation. In contrast, the repression of BARD1 in the presence of p53 decreased the transcriptional activation of p53 less than that of BARD1 siRNA alone (Fig. 5B).

BARD1, p53, and E6: are they related?

To test whether BARD1 regulates the expression of HPV E6, HPV E6 expression was tested by RT-PCR of RNA prepared from control siRNA-, BARD1 siRNA-, pcDNA3-HA-empty-, and pcDNA3-HA-BARD1-transfected CaSki cells. The repression of BARD1 by siRNA led to a marked increase in E6 mRNA level, raising it to a level significantly higher than that of endogenous E6. An increased E6 level was associated with the disruption of p53 protein, as demonstrated by immunoblot (Fig. 6A). Conversely, the overexpression caused by transfection of pcDNA3-HA-BARD1 led to a decrease in the E6 mRNA level. This resulted in an E6 mRNA level significantly lower than that observed after transfection of vector sequences. Importantly, overexpression of BARD1 resulted in increased p53 accumulation compared to that obtained with endogenous BARD1 (Fig. 6A). Control of independent gene expression was performed with primers against GAPDH.

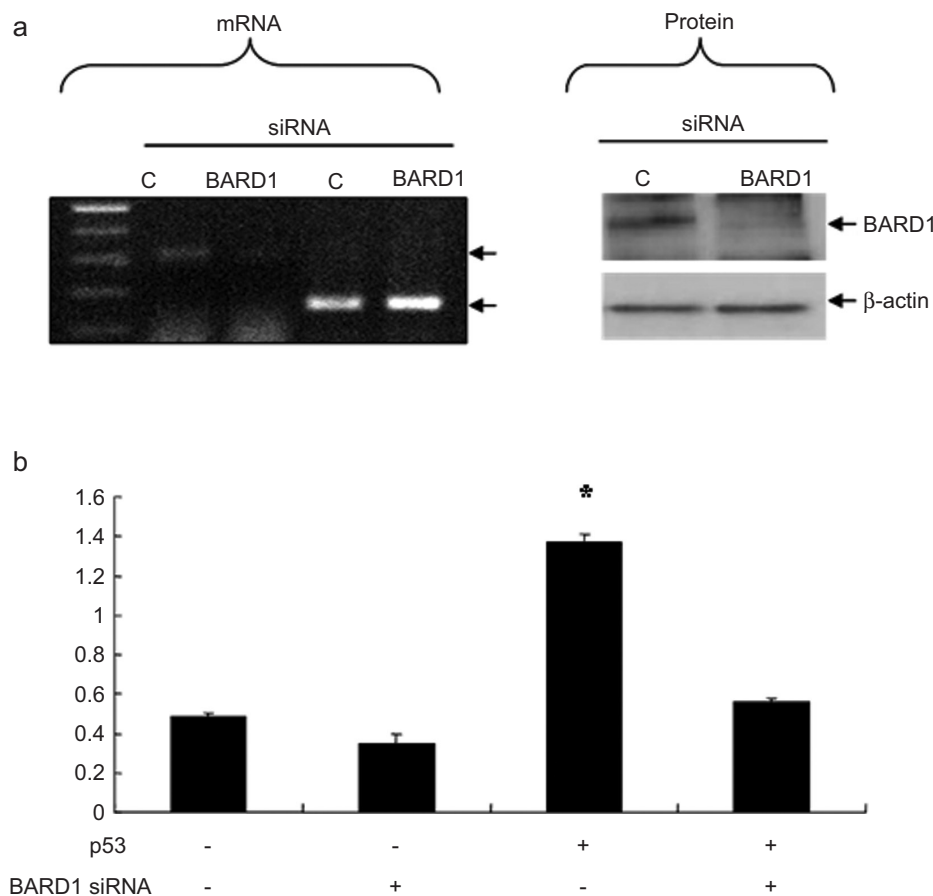


FIG. 5. The repression of BARD1 induces the reduction of p53-mediated transcriptional activation. (A) BARD1 mRNA and protein levels were subjected to RT-PCR and immunoblot analysis in control siRNA and BARD1 siRNA-transfected CaSki cells. Control siRNA was used for comparison with BARD1 siRNA. (B) A decrease of p53-mediated transcriptional activation by transfection of BARD1 siRNA in CaSki cells. CaSki cells were transfected with a combination of p53 and BARD1 siRNA together with p21^{WAF1} promoter-CAT reporter plasmid. Data represent the mean of at least three independent transfections. Fold stimulation was calculated by dividing amount of CAT enzyme of treated cells, as determined by CAT ELISA, by that of untreated control cells. These experiments were performed in triplicate (**p* < 0.05).

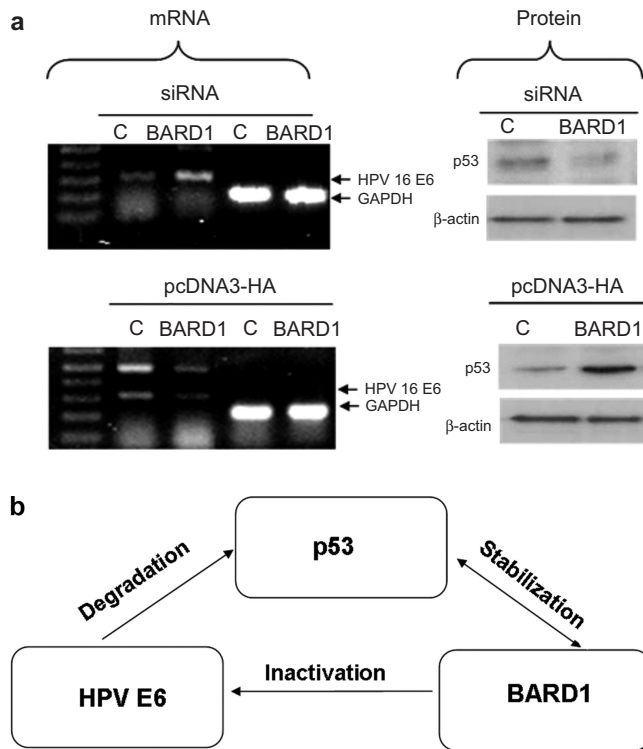


FIG. 6. The relationships among BARD1, p53, and E6. (A) HPV 16 E6 expression was tested by RT-PCR of RNA prepared from control siRNA⁻, BARD1 siRNA⁻, pcDNA3-HA-empty⁻, and pcDNA3-HA-BARD1-transfected CaSki cells. The p53 level was demonstrated by immunoblot analysis. The control of independent gene and protein expression was performed with GAPDH and β-actin. (B) The predictable model for the relationships among HPV E6, p53, and BARD1.

Discussion

BARD1 was originally identified through its interaction with BRCA1, with which it has a closely related domain structure. Both proteins possess an N-terminal RING-finger motif and two BRCT domains present in numerous proteins that are involved in DNA repair and cell cycle regulation. The functionally important BARD1/BRCA1 heterodimer formation is mediated by the RING-finger motifs and has also been shown to markedly increase the stability of both proteins (Wu *et al.*, 1996; Morris *et al.*, 2002). The finding of breast cancer is associated with mutations within the RING-finger domain of BRCA1, disrupting BRCA1/BARD1 interaction (Brzovic *et al.*, 2001), and the occurrence of *BARD1* missense mutations in breast cancer patients (Ghimenti *et al.*, 2002) implies the participation of BARD1 in BRCA1-mediated tumor suppression. BARD1, unlike BRCA1, also contains a centrally located sequence of three ankyrin repeats that are found in many of the proteins that are involved in transcriptional regulation (Sedgwick and Smerdon, 1999).

In yeast two-hybrid screening of a HeLa cDNA S3 library, we identified BARD1 as a partner that interacts with HPV 16 E6. Then, we assessed the proposed action of BARD1 in p53 stabilization and HPV E6 degradation action of BARD1 in cervical carcinoma cells. An increase of BARD1 protein affects p53 protein stabilization through a mechanism that most

likely involves direct BARD1-p53 protein-protein interaction (Irmingier-Finger *et al.*, 2001).

We have tested the ability of a panel of mutated HPV 16 E6 proteins to interact with BARD1 *in vitro*. Some E6 of the mutants that can bind to target p53 for degradation *in vitro* were able to dramatically reduce the level of BARD1 interaction. These included HPV 16 E6 mutants R39G, L50G, C66G, and C66G/C136G. It is of interest that BARD1 only interacts with high-risk HPV 16 and 18 E6, but not with low risk HPV 11 E6. Since the E6 protein contains two zinc-fingers composed of four metal-binding motifs (C-X-X-C), the disruption of the zinc-finger by mutations results in destabilization of the E6 structure, which in turn prevents it from binding to p53. Similarly, we observed that these mutations of E6 protein also inhibit the interaction between E6 and BARD1.

The p53 protein expressed within mammalian cells functions as a potent activator of transcription. Reconstitution of transcriptional activation by p53 in a cellular system should be a suitable manner by which to assess DNA binding and transcriptional activation activity regardless of posttranslational modifications and other influences that are inevitable when p53 is studied in the context of its regulatory network in mammalian cells. We were able to detect the effect of BARD1 on p53-dependent reporter gene activation by both the overexpression and repression of BARD1. BARD1 regulates p53 transcriptional activation regardless of the presence of E6; the overexpression of BARD1 led to an increase in the transcriptional activation of p53 regardless of the presence of E6. Conversely, the repression of BARD1 led to a decrease of that of p53. We were able to observe that the transcriptional activation of p53 after transfection with both BARD1 and p53 was higher than that after transfection with either BARD1 or p53 alone. Therefore, it could be expected that BARD1 collaborated with p53 and stabilized p53 against HPV infection.

Based on these results, we could make inferences regarding the relationships among HPV E6, p53, and BARD1. The best-characterized property of HPV E6 is its binding to the p53 tumor suppressor, which leads to degradation of the cellular protein via the ubiquitin pathway. The role of p53 is to safeguard the integrity of the genome by inducing cell cycle arrest or apoptosis in the presence of DNA damage. Therefore, when the E6 protein inactivates p53, it leads to chromosomal instability and to an increased probability of an HPV-infected cell evolving toward malignancy. The role of BARD1 is to increase and stabilize the p53 posttranslation. Conversely, BARD1 leads to E6 inactivation (Fig. 6B).

Our data, which involved the biological functions of the relationships among the three genes, HPV E6, p53, and BARD1, suggest that BARD1 may have important roles as a p53 co-suppressor in cervical carcinogenesis.

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