Mutations in BRCA1 are responsible for nearly all of the hereditary ovarian and breast cancers, and about half of those in breast cancer-only kindreds. The ability of BRCA1 to transactivate the p21 promoter can be inactivated by mutation of the conserved BRCA1 C-terminal (BRCT) repeats. To explore the mechanisms of this BRCA1 function, the BRCT repeats were used as bait in a yeast two-hybrid screen. A known protein, CtIP, a co-repressor with CtBP, was found. CtIP interacts specifically with the BRCT repeats of BRCA1, both in vitro and in vivo, and tumor-derived mutations in this region abolished these interactions. The association of BRCA1 with CtIP was also abrogated in cells treated with DNA-damaging agents including UV, γ-irradiation, and adriamycin, as a response correlated with BRCA1 phosphorylation. The transactivation of the p21 promoter by BRCA1 was diminished by expression of exogenous CtIP and CtBP. These results suggest that the binding of the BRCT repeats of BRCA1 to CtIP/CtBP is critical in mediating transcriptional regulation of p21 in response to DNA damage.

Mutations in BRCA1 are responsible for nearly all of the hereditary ovarian and breast cancers, and about half of those in breast cancer-only kindreds (1–3). How BRCA1 inactivation leads to tumor formation remains unclear. Studies of homozygous mutation of Brca1 in mice showed a phenotype of early embryonic lethality (4–6). Interestingly, Brca1−/− mouse embryonic stem cells are hypersensitive to ionizing radiation and hydrogen peroxide, and defective in transcription-coupled repair of oxidative DNA damage (7). An extension of development to embryonic day 11–12 was observed in Brca1−/− mice carrying additional p53 or p21null null mutations (8, 9). A role for BRCA1 in transcription regulation was provided by observations showing that expression of p21, a known target for p53 transcriptional activation, is increased significantly in Brca1 mutant embryos (8). Consistent with this observation, wild-type, but not mutant BRCA1, was able to transactivate the expression of p21 and inhibit cell cycle progression from G1 into S phase in human cells (10). Taken together, these results suggested that BRCA1 may have a role in the DNA repair process involving p21 and p53 expression. However, the molecular basis for these observations is largely unknown.

The C terminus of BRCA1 contains a transcription activation region (10–13) and two conserved BRCT repeats frequently found in proteins involved in DNA repair and cell cycle regulation (14–16). Although the general function of the BRCT motif is unclear, several lines of evidence suggest that it may be involved in protein-protein interactions (17, 18). Here, we report specific interactions between CtIP and the BRCT repeats of BRCA1 both in vitro and in vivo. Apparently, complex formation of BRCA1, CtIP and CtBP plays an important role in the regulation of p21 expression.

EXPERIMENTAL PROCEDURES

Plasmid Constructs—The pAS-BRCT plasmid was generated by polymerase chain reaction amplification of nucleotides 4898–5592 of BRCA1 using pBSK-BRCA1a (19) as the template and the following primers: 5′-CCGGAATTCGCGGAGGAGGACAAGATTGA-3′ and 5′-ATAGTAGATCTGAGCTGGTCGGGATG-3′. The 0.7-kilobase polymerase chain reaction product was cloned into the EcoRI/BamHI sites of pAS2–1 vector (CLONTECH, Palo Alto, CA). The pGST-BRCT plasmid was constructed by digestion of pAS-BRCT with EcoRI, Klenow fill-in, and ligation of BamHI linkers (New England Biolab, Beverly, MA). The construct was then digested with BamHI to release the BRCT fragment and subsequently cloned into BamHI site of pGEX-2T vector (Amersham Pharmacia Biotech). The GST-BRCT mutant was engineered using site mutagenesis kit (Stratagene, La Jolla, CA) with the following primers: 5′-CCAGGAGCTTGAACCTCATAGCATA-3′ and 5′-GGTCCAGCTCTCCCTTGAG-3′. pGAL4-BRCT was constructed by digestion of pGST-BRCT with BamHI to release the BRCT fragment and subsequently cloned into the BamHI site of pM2 vector (20). The pGAL4-BRCT mutants, including A1708E, P1749R, and Y1853term, were generated by site-directed mutagenesis with the following primers: 5′-CAGGAGCTTGAACCTCATAGCATA-3′ and 5′-GGTCCAGCTCTCCCTTGAG-3′; pGAL4 term was constructed by digestion of pGST-BRCT with BamHI to release the BRCT fragment and subsequently cloned into the BamHI site of pM2 vector (20). The pGAL4-BRCT mutants, including A1708E, P1749R, and Y1853term, were generated by site-directed mutagenesis with the following primers: 5′-CA

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transactivation activity, 50 μM of 3-amino-1,2,4-triazole was used in the screening to reduce the background.

In Vitro Binding Assay—Bacterially expressed and purified GST or GST fusion proteins were incubated with in vitro synthesized [35S]methionine-labeled CtIP, BRCA1, or CtIP and BRCA1 proteins as described (25).

Mammalian Two-hybrid Assay—Human kidney 293 cells were used in this assay as described (20). The expression of GAL4-X fusion proteins was verified by straight Westerns using a specific antibody that recognizes the GAL4 DNA-binding domain (Santa Cruz Biotechnology, Inc. Santa Cruz, CA).

Immunoprecipitation and Western Blot Analyses—Cells were lysed in lysis 250 buffer and immunoprecipitated as described (24). The immunoprecipitates were separated by SDS-polyacrylamide gel electrophoresis and analyzed by immunoblotting.

Cell Transfection and Luciferase Assay—Human kidney 293 cells were transfected with 0.5 μg of pWWW-luc or pG5E-luc, 0.5 μg of pSV40-β-gal, and 2 μg of each different plasmid DNA as indicated (control vector pcDNA3.1 was used to bring the final amount of DNA to 10 μg) using calcium phosphate/DNA co-precipitation method. Luciferase activity was measured 48 h after transfection as described (26). For adriamycin treatment, HCT116 cells were incubated with adriamycin (0.2 μg/ml) for 24 h before harvest.

RESULTS

CtIP Interacts Specifically with Wild-type BRCT Repeats of BRCA1—To explore the potential function of the BRCT repeats of BRCA1, we used them as bait in a yeast two-hybrid screen for interacting proteins (24). One of the 20 clones isolated encodes amino acids 17 to 713 of the known protein CtIP (Fig. 1A), which was also identified by others using a different approach (27, 28).

To test whether CtIP directly binds to the BRCT repeats, an in vitro transactivation assay using GST-fusion proteins was performed. The BRCT repeats that served as the bait in the above screen and a mutant containing a familial 1853 tyrosine nonsense mutation were fused with GST (GST-BRCTΔ, Fig. 1B). Bacterially expressed and purified GST-BRCT, but not GST-BRCTΔ or GST (Fig. 1C), can bind to in vitro synthesized [35S]methionine-labeled CtIP protein (Fig. 1D, lane 3, compare lanes 2 and 4).

To ascertain whether CtIP and the BRCT repeats of BRCA1 can interact in cells, a mammalian two-hybrid assay (20) was
performed. Full-length CtIP was fused to the VP16 transactivating domain of herpesvirus in an expression vector (pVP16-CtIP), and a panel of expression vectors encoding the DNA binding-domain of GAL4 fused to either wild type (GAL4-BRCT, Fig. 1E) or mutated (GAL4-BRCTM, Fig. 1E) BRCT repeats of BRCA1 were constructed. The BRCT mutants contain individual alterations identified in familial breast cancers including missense (A1708E, first BRCT repeat; and P1749R, spacer region) and nonsense (Y1853Term, second repeat) mutations. Human kidney 293 cells were co-transfected with a GAL4-responsive luciferase (pG5E-luc) and β-galactosidase (pSV40-β-gal) reporters, expression plasmids for either GAL4 or GAL4-BRCT (wild type or mutant), and VP16 or VP16-CtIP. A significant increase in luciferase activity was observed upon co-expression of wild-type GAL4-BRCT and VP16-CtIP (Fig. 1E, lanes 5 and 6). In contrast, no obvious activity was observed upon co-expression of GAL4 or wild-type GAL4-BRCT with VP16 (Fig. 1E, lanes 1–4), or the mutated BRCT repeats in GAL4-BRCTM with VP16-CtIP (Fig. 1E, lanes 7–12). Because the cells transfected with GAL4, GAL4-BRCT and GAL4-BRCTM (Fig. 1F, lanes 2–6) expressed these fusion proteins at comparable levels, the reduction of luciferase activity was not attributable to lack of protein expression.

Identification of Cellular CtIP Protein—To study the in vivo interactions of endogenous BRCA1 and CtIP, mouse polyclonal antibodies recognizing CtIP (C21) were generated using an antigen consisting of GST translationally fused with amino acids 324–537 of CtIP. [35S]methionine-labeled human colon carcinoma cell (HCT116) lysates immunoprecipitated with C21 identified a band (Fig. 2A, lane 2) whose mobility was consistent with the predicted molecular mass (125 kDa) of CtIP (21). A band of similar size was not detected in the immunoprecipitates of pre-immune serum (Fig. 2A, compare lanes 1 and 2). Pre-incubation of the antiserum with GST-CtIP, but not GST resulted in specific depletion of the 125-kDa band (Fig. 2A, lanes 3 and 4). Reprecipitation of the immunoprecipitates by a second incubation with anti-CtIP antibodies resulted in a specific 125-kDa band (Fig. 2A, lane 5). Based on these results, we concluded that the 125-kDa protein is the cellular CtIP protein.

Co-immunoprecipitation of CtIP and BRCA1 in Vivo—The in vivo interaction between CtIP and BRCA1 was further examined in HCT116 and breast cancer cells, HCC1937 (29), by co-immunoprecipitation. HCC1937 cells contain an insertion of cytosome at nucleotide 5382 of BRCA1 that generates a frameshift at amino acid 1794, which stops translation at 1829 (29). Anti-BRCA1 monoclonal antibody 6B4 (30), but not control antibody against p84 (N5), a nuclear matrix protein (31), specifically immunoprecipitated a 220-kDa protein in HCT116 cell lysates (Fig. 2B, compare lanes 1 and 2). Consistent with the 5382insC mutation in BRCA1, a faster migrating product was detected in HCC1937 cell lysates (Fig. 2B, lane 3). This protein is likely to be the HCC1937 BRCA1 product because it cannot be recognized by C-20 antibodies (against amino acids 1843–1862 of BRCA1), which readily immunoprecipitated a 220-kDa protein from HCT116 (Fig. 2B, compare lanes 5 and 4). Furthermore, using anti-BRCA1 6B4, but not control antibody N5, CtIP was co-immunoprecipitated with BRCA1 in HCT116 cell lysates (Fig. 2B, compare lanes 1’ and 2’). No detectable CtIP was present in the 6B4 immunoprecipitates from HCC1937 cell lysate (Fig. 2B, lane 3’). Consistent with this result, BRCA1 was reciprocally co-immunoprecipitated with anti-CtIP C21 antibody from HCT116 cell lysate, but not from HCC1937 cell lysate (Fig. 2B, compare lanes 8 and 7). Levels of CtIP immunoprecipitates detected from both cell lines were comparable (Fig. 2B, lanes 7’ and 8’). These data indicated the existence of an in vivo complex of BRCA1 and CtIP, that, in HCC1937 cells, is disrupted presumably by an altered C terminus lacking intact BRCT repeats.

Dissociation of CtIP from BRCA1 upon Treatment with DNA-damaging Agents—Since BRCA1 has a potential role in DNA repair, its interaction with CtIP may mediate cellular responses to DNA damage. Therefore, it is possible that their interaction might be altered upon treatment of cells with DNA-damaging agents. To address this possibility, HCT116 cells were treated with UV, γ-rays, or adriamycin, and the cell lysates were immunoprecipitated with anti-BRCA1 antibody (6B4). Consistent with previous data (32, 33), a slower migrating form of phosphorylated BRCA1 was detected after treatment of the cells with DNA-damaging agents (Fig. 3A, lanes 3–5 compare with lane 2). Importantly, the association of CtIP was undetectable in the BRCA1 immunoprecipitates subse-
transiently transfected with pRcCMV-CtIP to overexpress the T7-tagged CtIP full-length protein. As shown, anti-T7-tag antibody can co-immunoprecipitate the full-length CtIP protein, cellular CtIP, and BRCA1 (Fig. 4B, lane 2), but not the control antibody (lane 1). In the reciprocal experiment, anti-BRCA1 antibody can bring down cellular BRCA1, CtIP and T7-tagged CtIP (Fig. 4B, lane 4). These results suggest that CtBP, CtIP, and BRCA1 can form a complex.

Repression of BRCA1-dependent Transactivation of the p21 Promoter by CtIP and CtBP—Previous studies (10, 12) showed that BRCA1 was able to transactivate p21 expression. Formation of the CtBP, CtIP, and BRCA1 complex predicts that ectopic expression of CtIP or CtBP may affect BRCA1-dependent transactivation of the p21 promoter. To test this hypothesis, transient transfections of human 293 cells were performed with a p21 promoter-luciferase reporter plasmid pWWW-luc, pSV40-β-gal transfection control plasmid, and combinations of BRCA1, CtIP, and CtBP expression vectors. The expression of BRCA1 resulted in a 5-fold activation of the p21 promoter compared with empty vector alone. Co-expression of CtIP moderately inhibited BRCA1-dependent transactivation of the p21 promoter. However, co-expression of CtIP and CtBP repressed p21 promoter activity to background levels (Fig. 5A). Interestingly, co-expression of CtBP and BRCA1 also resulted in significant repression of p21 promoter, which was likely due to the abundance of endogenous CtIP in cells. Likewise, the modest inhibition of BRCA1-dependent transcription by CtIP may be due to the recruitment of cellular CtBP. Overexpression of CtBP alone does not have an effect on p21 promoter (Fig. 5A). The repression of BRCA1-dependent transactivation of p21 promoter by CtBP and CtIP depends on their association with BRCA1, because overexpression of CtIP and CtBP cannot repress the transactivation of GAL4 promoter (pG5E-Luc) by the GAL4-VP16 hybrid (Fig. 5B). These experiments suggest that a potential biological function of the BRCA1/CtIP interaction is to repress target promoters through contacts with the CtBP co-repressor.

**DISCUSSION**

The BRCT repeats were first identified as a highly conserved structural domain among more than 50 nonorthologous proteins, many of which are involved in DNA repair and cell cycle checkpoint control (14–16). Familial mutations have been frequently found in the BRCT repeats of BRCA1, suggesting that the function of the BRCT repeats of BRCA1 is important.

**FIG. 3.** Dissociation of BRCA1 from CtIP upon DNA-damaging agents treatment. A, BRCA1/CtIP interactions were altered in response to DNA damage. HCT116 cells treated with UV radiation (1 mJ/cm²), γ-irradiation (10 Gy) were harvested 1 h subsequently. Cells treated with adriamycin (0.2 mg/ml) were harvested 24 h later. Lysates from untreated and treated HCT116 cells were immunoprecipitated with anti-p84 control antibody (lane 1) or anti-BRCA1 antibody-6B4 (lanes 2–5), separated by SDS-PAGE, and transferred to membranes. In the upper panel, the membrane was probed with anti-BRCA1 antibody, 6B4. Note the appearance of slower migration forms of BRCA1 after treatment (lanes 3–5, compare with lane 2). In the lower panel, the membrane was probed with anti-CtIP antibody, C21. CtIP was not detected in the anti-BRCA1 immunoprecipitates in lysates prepared from treated cells (lanes 3–5) but is readily detected in untreated cells (lane 2). B, expression levels of CtIP in cells treated with DNA-damaging agents was not altered. Aliquots of cell lysates used for the above immunoprecipitations were assayed by straight Westerns using the indicated antibodies. The upper panel shows a membrane probed with anti-CtIP antibody. Note that the levels of CtIP expression were comparable before or after treatment. As a protein loading control, the lower panel shows a membrane probed with anti-p84 antibody.

**FIG. 4.** BRCA1 binds to CtIP through CtIP. A, in vitro synthesized [35S]methionine-labeled protein from full-length wild-type (lane 1) or mutated CtIP (PLDLS-LASQC, lane 5), was incubated with GST (lanes 2 and 6), GST-BRCT (lanes 3 and 7), or GST-CtBP (lanes 4 and 8). GST-CtBP binds to wild-type, but not the mutated CtIP (compare lanes 4 and 8). B, HCT116 cells were transfected with pRcCMV-CtBP plasmid. The cell lysates were immunoprecipitated with 8G11 (anti-GST monoclonal antibody, lanes 1 and 3), α-T7 (anti-T7-tag monoclonal antibody, lane 2), or 6B4 (anti-BRCA1 monoclonal antibody, lane 4). The membranes were probed with 6B4, C21, or α-T7 antibody as indicated. T7-tagged CtBP full-length protein is indicated by an arrow. The IgG heavy chain is marked by an asterisk.
BRCA1/CtIP complex in p21 regulation

for the BRCA1 tumor suppression function. Using BRCT repeats as bait, we have identified a BRCA1-associated protein, CtIP. CtIP interacts specifically with the BRCT repeats of BRCA1 both in vitro and in vivo, and tumor-derived mutations in BRCT repeats abolish this specific interaction. The three tumor-derived mutations A1708E, P1749R, and Y1853C were found in E1A and several viral proteins (22, 35, 36). BRCA1 does not have a PLDLS motif in CtIP as the binding site for CtBP (22). This motif was also found in E1A and several viral proteins (22, 35, 36). BRCA1 does not have a PLDLS motif, and cannot bind to CtBP directly. Apparently, CtIP has different binding motifs for CtBP and BRCA1 respectively, allowing dissociation of CtIP and BRCA1 is correlated with the hyperphosphorylation of BRCA1 upon treatment with DNA-damaging agents. These results suggest that CtIP/CtBP may negatively regulate the transcription activity of BRCA1 on the p21 promoter. However, whether BRCA1 transactivates p21 promoter directly or binds to additional transcription factors remains to be explored.

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