The C-terminal (BRCT) Domains of BRCA1 Interact in Vivo with CtIP, a Protein Implicated in the CtBP Pathway of Transcriptional Repression*

(Received for publication, June 22, 1998, and in revised form, August 3, 1998)

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The BRCA1 tumor suppressor encodes a polypeptide with two recognizable protein motifs: a RING domain near the N terminus and two tandem BRCT domains at the C terminus. Studies of tumor-associated mutations indicate that the RING and BRCT sequences are required for BRCA1-mediated tumor suppression. In addition, recent work has shown that BRCA1 is a potent regulator of RNA transcription and that the BRCT domains are also essential for this activity. Therefore, we used the Sos recruitment system to screen for proteins that bind this critical region of BRCA1. Our results show that the BRCT domains interact in vivo with CtIP, a protein originally identified on the basis of its association with the CtBP transcriptional co-repressor. This finding suggests that BRCA1 regulates gene expression, at least in part, by modulating CtBP-mediated transcriptional repression. Moreover, the in vivo interaction between BRCA1 and CtIP is completely ablated by each of three independent tumor-associated mutations affecting the BRCT motifs of BRCA1. These results indicate that the BRCA1-CtIP interaction may be required for tumor suppression by BRCA1.

Several lines of evidence suggest that BRCA1 modulates gene expression at the level of RNA transcription. First, the BRCT sequences of BRCA1 can activate RNA transcription when expressed as fusions with a heterologous DNA-binding domain (9, 10). Second, wild type BRCA1 polypeptides co-purify from whole cell extracts with the holoenzyme of RNA polymerase II (11). Third, wild type BRCA1 can potentiate the transcription of certain reporter genes when overexpressed in mammalian cells (12–14). Remarkably, each of these properties is abrogated by tumor-associated alterations of the BRCT domains, such as the A1708E and M1775R missense mutations and a nonsense mutation that deletes the C-terminal 11 amino acids of BRCA1 (Y1853A) (9–14). Thus, if BRCA1 does function in transcriptional regulation, the BRCT sequences are likely to have a central role in this process.

The BRCT domain is found in more than 50 distinct proteins, many of which have defined roles in the cellular response to DNA damage (6, 15, 16). Although a common molecular function for this domain has not been uncovered, the BRCT sequences of some proteins serve as sites of protein-protein interaction (17–19). Therefore, we used the Sos recruitment system (SRS), a new form of yeast two-hybrid analysis (20), to screen for protein interactions involving the BRCT sequences of BRCA1. Several candidate BRCA1-interacting proteins were identified; our data show that one of these, the CtBP-interacting protein (CtIP), readily associates with BRCA1 in mammalian cells. CtIP was recently described as a polypeptide that binds CtBP (21), a transcriptional co-repressor used by the adenoviral E1A oncoprotein (22, 23) and several diverse DNA-binding factors, including the Drosophila Knirps, Snail, and Hairy proteins (24, 25). Thus, BRCA1 may regulate gene expression by targeting the CtBP pathway of transcriptional repression. Significantly, the interaction between CtIP and BRCA1 is abolished by tumor-associated missense mutations in the BRCT domains of BRCA1, indicating that formation of a stable BRCA1-CtIP complex may be required for BRCA1-mediated tumor suppression.

EXPERIMENTAL PROCEDURES

Expression Vectors and cDNA Libraries for SRS Screening—An expression plasmid encoding the SRS bait was produced by inserting cDNA sequences encoding the SZ segment of human BRCA1 (amino acids 1528–1863) into pAD-FLAG, a yeast shuttle vector that contains coding sequences for an N-terminal moiety of human Sos (residues 1–1070). The pAD-FLAG vector was itself generated in two steps using the Sos-coding and synthetic polylinker sequences. First, a synthetic polylinker fragment containing the Sos-coding and synthetic polylinker sequences was excised from the resultant vector and inserted into the dIII/HindIII site of pYes-5HIN. 

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*This work was supported by Grants CA76334 (to R. B.) and CA60650 (to A. M. B.) from the National Cancer Institute. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† Recipient of an Academic Lectureship from Samuel and Miriam Wein.

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Not 1 sites of pADNS. To prepare cDNA libraries for SRS screening, polyadenylated RNA from HBL-100 cells was converted to cDNA using the Superscript™ Plasmid System (Life Technologies, Inc.) and inserted into yeast shuttle vectors that contain coding sequences for the v-Src myristoylation signal. Two libraries of HBL-100 cDNA were prepared using different vectors: one library contains ~30 million independent transformants in the pYes-Mpoly(A) vector (the ne-2 library) and the other has ~7 million independent transformants in pYes-Sal (the ns-1 library). The pYes-Mpoly(A) vector has been described (20), and pYes-Sal was produced by inserting a synthetic polylinker into the EcoRI/NotI sites of pYes-Mpoly(A).

SRS Screening—SRS screening in yeast was conducted essentially as described (20) except that transformation of cdc25–2 cells with the bait and library plasmids was done successively rather than simultaneously. Plasmid DNA was recovered from each colony that displayed galactose-dependent growth at the restrictive temperature (36 °C). In addition to bait-interacting clones, SRS screening also selects for library plasmids that encode certain components of the Ras signaling pathway (20). Because TC21 is the most abundantly expressed Ras protein in HBL-100 cells, library plasmids encoding TC21 were identified by dot blot DNA hybridization and eliminated from further consideration. The remaining library plasmids were then subjected to nucleotide sequence analysis; this step eliminated plasmids encoding other components of the human Ras signaling pathways (e.g. H-Ras, CDC25, etc.) or containing cDNA inserts that are out-of-frame with the coding sequence for the v-Src myristoylation signal. To confirm the interaction in yeast, each of the remaining plasmids was then co-transfected into cdc25–2 cells together with a vector encoding either the original Sos-SZ bait or the Sos domain alone; those clones that interacted with the Sos-SZ hybrid, but not the Sos domain, upon re-transfection were deemed to be “positive.” Eighty positive clones representing fourteen independent cDNA clones from nine different mRNAs were obtained by screening ~25 million library transformants.

Mammalian Two-hybrid Analysis—Expression plasmids that encode hybrid polypeptides were constructed by inserting cDNA fragments into the pCMV-GAL4 vector (26) or the pVP-FLAG5, 6, or 7 vectors (27). Mammalian two-hybrid assays were conducted in 293 cells as described (27). To verify expression of the GAL4-X hybrids, lysates of each transfected culture were fractionated by SDS-PAGE and immunoblotted with a Gal4-specific rabbit antiserum (Upstate Biotechnology, Inc.).

Co-immunoprecipitation Analysis—Expression vectors for human PCTAIRE-1 were provided by Sander van den Heuvel (28). For co-immunoprecipitation analysis, each 100-mm plate of 293 cells was transfected with 7.0 μg of the expression plasmid and 0.5 μg of the pCMV/β control plasmid (CLONTECH) using the calcium phosphate transfection system (Life Technologies, Inc.) (26). Two days later, cell lysates were prepared in 0.5 mL of “high salt” Nonidet P-40 buffer (1 M NaCl, 10 mm Hepes, pH 7.6, 0.1% Nonidet P-40, 5 mM EDTA) containing protease inhibitors (2 μg/ml aprotinin, 2 μg/ml leupeptin, 1 μg/ml pepstatin, and 1 mM phenylmethylsulfonyl fluoride). 10 μl of each lysate were evaluated by immunoblotting with the HA-specific monoclonal antibody (12CA5) to confirm expression of the HA-tagged polypeptide. The remainder of each lysate was incubated with 16 μg of 12CA5 monoclonal antibody. After rocking at 4 °C for 2 h, 40 μl of staphylococcal protein A-Sepharose beads (20% slurry) (Amersham Pharmacia Biotech) were added to each lysate, and the mixture was rocked at 4 °C for an additional 2 h. The beads were then pelleted by centrifugation and washed four times in high salt Nonidet P-40 buffer with protease inhibitors and two times in “low salt” (250 mM NaCl) Nonidet P-40 buffer with protease inhibitors. Finally, the immunoprecipitates were examined by Western analysis (26) using antibody reagents that recognize either N-terminal (residues 1–304) (MS110 mouse monoclonal; Oncogene Research Products) (29) or C-terminal (residues 1844–1863) (C-20 rabbit polyclonal; Santa Cruz Biotechnology) sequences of human BRCA1.

RESULTS

Use of the Sos Recruitment System to Identify BRCA1-interacting Proteins—To identify proteins that interact with the BRCT domains of BRCA1, we used the SRS, a new form of yeast two-hybrid analysis that does not involve a transcripational readout (20). SRS screening exploits the ability of human Sos, a guanyl nucleotide exchange factor for Ras proteins, to rescue the growth defect of Saccharomyces cerevisiae strains bearing the temperature-sensitive cdc25–2 allele (30). To detect protein-protein interactions, cdc25–2 cells are co-transfected with plasmids encoding two hybrid polypeptides: one containing the N-terminal 1070 residues of Sos fused to protein X (the Sos-X hybrid) and the other containing the myristoylation signal of v-Src fused to protein Y (the Myr-Y hybrid). If proteins X and Y interact stably in vivo, then the Myr-Y hybrid will recruit Sos-X to the inner surface of the plasma membrane; the Sos moiety of Sos-X will then catalyze nucleotide exchange of membrane-bound Ras and restore the growth of cdc25–2 cells at the restrictive temperature (20).

For SRS screening we constructed a yeast expression plasmid that encodes Sos-SZ, a hybrid polypeptide containing sequences of human Sos (residues 1–1070) fused to the SZ segment of human BRCA1 (residues 1528–1863). Cdc25–2 yeast cells were then transfected with the Sos-SZ expression plasmid, and the synthesis of Sos-SZ polypeptides in these cells was confirmed by immunoblotting with a Sos-specific antiserum (data not shown). We also prepared cDNA libraries from HBL-100, an immortalized line of normal human mammary epithelial cells (31). These libraries, which encode hybrid polypeptides containing the myristoylation signal of v-Src fused to cDNA products of HBL-100, were used to transform cdc25–2 yeast cells that express the Sos-SZ bait. In all, 18 isolates that restored the growth of cdc25–2 cells in a BRCA1-dependent manner were obtained by screening approximately 25 million library transformants (see “Experimental Procedures”). Sequence analysis revealed that these isolates represent 14 independent cDNA clones derived from 9 distinct mRNAs. We obtained four independent cDNA isolates encoding the PCTAIRE-1 kinase (GenBank™ accession number X66363) and three encoding hSDP, the homolog of a single-strand DNA-binding protein from chickens (cSDP, accession number U68380). In addition, single isolates were obtained for the heat shock cognate 70-kDa protein (HSC70; accession number P11142), the CtBP-interacting protein (CtIP; accession number U72066), and five additional proteins that have not been described previously.

Analysis of the BRCA1-interacting Proteins in Mammalian Cells—The mammalian version of the conventional two-hybrid assay was used to ascertain whether any of the candidate BRCA1-interacting proteins associate with BRCA1 in mammalian cells (27, 32). For this purpose we constructed a mammalian expression plasmid that encodes “VP16-SZ,” a hybrid polypeptide containing the transactivation domain of herpesvirus VP16 fused to the SZ segment of BRCA1, and a panel of vectors encoding the DNA-binding domain of Gal4p fused to each of the nine candidate BRCA1-interacting proteins. The mammalian two-hybrid assay was then performed by transfecting human 293 cells with a Gal4p-responsive reporter gene and pairwise combinations of the appropriate expression vectors. Reporter gene expression was determined by measuring the luciferase activity in cell lysates from each transfected culture. As illustrated in Fig. 1, luciferase activity was not significantly induced by co-expression of the VP16-SZ hybrid with the DNA-binding domain of Gal4p (lane 1) or by co-expression of the Gal4-CtIP hybrid with the VP16 transactivation domain (lane 15). However, a dramatic increase in luciferase activity was elicited by co-expression of the VP16-SZ and Gal4-CtIP hybrids (lane 13). In contrast, none of the other candidate BRCA1-interacting proteins induced a measurable increase upon co-expression with VP16-SZ (lanes 3, 5, 7, 9, 11, 15, 17, and 19). Western analysis with a Gal4p-specific antiserum confirmed that each of the GAL4-X hybrids was expressed at levels comparable with or greater than that of GAL4-CtIP (data not shown). The BRCA1-CtIP interaction was also detected by mammalian two-hybrid analysis in SW480, a line of human colon carcinoma cells (data not shown). The inability of the
other eight proteins to interact with BRCA1 in the mammalian two-hybrid assay does not necessarily eliminate them as candidate BRCA1-associated proteins (26, 27). Nevertheless, these results indicate that CtIP forms a stable complex with BRCA1 in mammalian cells and that the interaction is mediated by sequences within the C-terminal 336 amino acids of BRCA1 (i.e. the SZ fragment).

CtIP is a polypeptide of 897 amino acids that was recently identified on the basis of its interaction with the C-terminal binding protein (CtBP) (21). Nucleotide sequence analysis revealed that the cDNA library clone recovered by SRS screening encodes the C-terminal 853 amino acid residues of human CtIP (data not shown). To confirm the ability of CtIP to associate with BRCA1 in mammalian cells, co-immunoprecipitation experiments were performed with lysates of transfected 293 cells. Therefore, 293 cultures were transfected with a series of expression plasmids, and the normalized luciferase activities obtained from each transfection are illustrated.

The BRCA1-CtIP Interaction Is Mediated by the BRCT Sequences of BRCA1—The data described above indicate that CtIP interacts with the SZ fragment of BRCA1 (amino acids 1528–1863), a region that includes the two tandem BRCT motifs (Fig. 3A). To define more specifically the sequence requirements for CtIP binding, smaller C-terminal segments of BRCA1 were tested for their potential to associate with CtIP in the mammalian two-hybrid assay (Fig. 3A). As shown in Fig. 3B, the ability to bind CtIP is retained by the CT segment of BRCA1 (residues 1651–1863) (lanes 3) but not by segments AZ and GZ (lanes 7 and 9, respectively), each of which lacks sequences from BRCT motif 1 (Fig. 3A). In separate experiments, the NP segment, which lacks sequences from BRCT motif 2 (Fig. 3A), was also unable to associate with CtIP (data not shown). Thus, the minimal BRCA1 sequence required for CtIP association (i.e. the CT segment; residues 1651–1863) corresponds almost precisely to the two tandem BRCT domains, indicating that both BRCT motifs are involved in the interaction with CtIP (Fig. 3A).

Tumorigenic BRCA1 Mutations: Their Effect on the BRCA1-CtIP Interaction—Tumor susceptibility in some families with hereditary breast cancer has been ascribed to subtle alterations in the BRCT sequences of BRCA1. These tumor-associated lesions include missense mutations that produce single amino acid substitutions in either the first (e.g. A1708E) or second (M1775R) BRCT motif, as well as a nonsense mutation that eliminates the C-terminal 11 amino acids of BRCA1 (Y1853X) (7, 8). To evaluate their effect on the BRCA1-CtIP interaction, GAL4-SZ hybrid polypeptides containing each of these lesions were tested for the ability to bind VP16-CtIP in the mammalian two-hybrid assay (Fig. 4A). As expected, the wild type GAL4-SZ hybrid readily interacts with VP16-CtIP (Fig. 4A, lane 3). However, this interaction is abolished by each of the three tumor-associated BRCA1 mutations (lanes 5, 7, and 9), despite the fact that the mutant GAL4-SZ polypeptides are expressed at levels equivalent or greater than that of wild type.
vivo association with CtIP. The individual segments of BRCA1 that were tested for interaction with CtIP (in vertical arrows) are represented by amino acids residues 1649–1736 and 1756–1855, respectively (15). The tumor-suppressive functions of the BRCT domain (denoted by vertical arrows) are shown beneath the map. These include segments SZ (BRCA1 residues 1528–1863), CT (residues 1651–1863), AZ (residues 1669–1863), GZ (residues 1710–1863), and NP (residues 1651–1785). β, mammalian two-hybrid analysis of the interaction between CtIP and defined segments of BRCA1. Each dish of 293 cells was co-transfected with the G5LUC reporter plasmid, the β-galactosidase control plasmid, and the two indicated expression vectors. The VP16 expression vector encoded either the parental VP16 transactivation domain (denoted by + in the VP16 column) or the VP16-CtIP hybrid. The GAL4p expression vector encoded either the parental GAL4p DNA-binding domain (denoted by + in the GAL4 column) or the GAL4-CtIP hybrid. The VP16 expression vector encoded either the parental VP16 transactivation domain (denoted by + in the VP16 column) or a VP16-hybrid polypeptide containing the SZ, CT, AZ, or GZ segments of BRCA1 (see panel A).

GAL4-SZ (Fig. 4B). These results demonstrate that tumorigenic lesions of the BRCT domain prevent the association of BRCA1 and CtIP.

Discussion

Although BRCA1 may play a central role in the cellular response to DNA damage (1–4), the biochemical mechanisms by which BRCA1 exerts its effects are not understood. Through its association with known repair proteins such as HsRad51 (2, 3), BRCA1 may participate directly in the biochemical processes that mend damaged DNA. In addition, however, BRCA1 may also serve as a sensor or signal transducer that detects DNA damage and helps to coordinate the appropriate cellular response. Consistent with this, recent studies show that BRCA1 has the potential to regulate RNA transcription (9–14). Significantly, the transcriptional functions of BRCA1 are obviated by subtle alterations in the BRCT domains, including the A1708E and M1775R missense mutations (9–14). Also, gene transfer experiments have shown that cell growth can be inhibited by overexpression of truncated BRCA1 polypeptides that retain the BRCT domains (34, 35) and that growth inhibition by these polypeptides is mitigated by the A1708E and M1775R mutations (34). Thus, the BRCT sequences of BRCA1, as well as the transcriptional regulatory functions ascribed to these sequences, may well be essential for BRCA1-mediated tumor suppression.

Our data suggest that transcriptional regulation by BRCA1 is mediated by its association with CtIP, a protein that was recently identified on the basis of its ability to bind the CtBP transcriptional corepressor (21). CtBP was itself isolated as a transcriptional repressor that interacts with C-terminal sequences of the adenoviral E1A oncoprotein (22). This region of E1A includes a short motif of five amino acids (PLDLS) that is required for CtBP binding (22, 33). Deletion or mutation of the CtBP-binding motif dramatically enhances the in vitro transformation potential and in vivo tumorigenicity of E1A, implying that this motif normally serves to repress E1A-mediated oncogenesis (33). Moreover, the CtBP-binding sequence of E1A is a potent repressor of RNA transcription when targeted to a promoter by fusion with a heterologous DNA-binding domain (23). Significantly, the tumor-repressing and transcriptional repression properties of the E1A C-terminal region correlate with its ability to bind CtBP (22, 23, 33).

Recent studies of Drosophila support the notion that CtBP functions as a co-repressor, i.e. a transcriptional repressor that is recruited to specific target genes by virtue of its association with an appropriate DNA-binding transcription factor. In particular, Nibu et al. (24) have shown that the transcriptional...
repression domains of Knirs and Snail have sequences resembling the PLDLS motif of E1A and that these sequences mediate a functional interaction with the Drosophila ortholog of CtBP. Likewise, Poortinga et al. (25) reported that transcriptional suppression by the Hairy protein also involves in vivo association with Drosophila CtBP through a PLDLS-like sequence. Notably, each of these CtBP-interacting proteins belongs to a different class of DNA-binding transcription factor; DNA recognition by the Hairy, Knirs, and Snail proteins is mediated, respectively, by a helix-loop-helix domain, a nuclear receptor zinc-binding motif, and conventional (i.e. Tramtralike) zinc fingers (24, 25). Thus, by using a PLDLS-like motif to recruit CtBP, DNA-binding transcription factors that are otherwise unrelated can repress gene expression by interaction with a common co-repressor.

CtIP was recently identified as a human CtBP-interacting polypeptide that does not have obvious sequence homology with other known proteins (21). Nonetheless, CtIP does possess a CtBP-binding sequence (PLDLS) identical to that of E1A, and mutation analysis indicates that this sequence is required for the interaction of CtIP with CtBP (21). Thus, CtIP belongs to a family of diverse proteins that employ a common PLDLS-like motif to bind the CtBP co-repressor. Although some members of this family possess recognizable DNA-binding motifs (e.g. Hairy, Knirs, and Snail), others, such as viral E1A and cellular CtIP, do not. The effects of CtIP on the DNA repair, transcription, and tumor suppression properties of BRCA1 remain unclear. The function of CtIP with respect to the CtBP pathway of transcriptional repression is also not understood. On one hand, CtIP may serve as a downstream effector of CtBP that represses RNA transcription. On the other hand, CtIP may function as a regulator of CtBP that either promotes or inhibits CtBP-mediated transcriptional repression. Any one of these alternatives might account for the transcriptional activation properties displayed by the BRCT sequences of BRCA1 (9, 10) or the ability of BRCA1 to modulate the expression of certain reporter genes (12–14).

Acknowledgments—We are especially grateful to Ying Xia for invaluable advice and to Sander van den Heuvel for providing the PCTAIRE-1 expression vectors.

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