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13. ABSTRACT *(Maximum 200 Words)*

Cancer-predisposing mutations in BRCA1 account for a large proportion of familial breast cancer. The BRCT domain of BRCA1 is important for BRCA1 function in DNA repair and transcriptional activation. When tethered to chromosomal DNA, this region of BRCA1 is capable of inducing changes in chromatin structure. Using yeast genetic approaches as proposed in the application, we identified several interesting proteins that physically interact with the BRCT domain of BRCA1 and facilitate BRCA1 function in chromatin reorganization. Furthermore, we provide functional data that suggest a cooperative action of BRCA1 and its cofactors in transcriptional regulation and tumor suppression. Thus, the identification and characterization of these BRCA1 cofactors accomplish the original research goals and lay a solid foundation for future study to elucidate the BRCA1-mediated functional pathway in suppression of breast cancer development.

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Table of Contents

INTRODUCTION

Germ line mutations in *BRCA1* confer elevated risks in the development of familial breast and ovarian cancers (1) (2). *BRCA1* encodes a 1863-amino acid protein with a highly conserved RING finger domain at the amino terminus and two BRCT repeats at the extreme carboxyl terminus. While most disease-associated mutations of BRCA1 are predicted to result in gross truncation of the protein, 5-10% of the cancer-predisposing mutations cause single amino acid substitutions (3), many of which are located in the RING domain or BRCT repeats. It is generally assumed that both types of mutations lead to loss of the biological functions of the protein, however, several genotype-phenotype correlation studies suggest that BRCA1 mutations at different locations of the gene may confer different BRCA1-dependent cancer risks (4) (5).

Intense research in the past several years has implicated BRCA1 in regulation of multiple nuclear processes including DNA repair and transcription (6) (7) (8) (2) (9) (10). For example, BRCA1-deficient mouse and human cells are hypersensitive to ionizing radiation, due to defects in transcription-coupled repair of oxidative DNA damage, as well as double strand break-induced homologous recombination (11) (12) (13) (14) (15). In addition, BRCA1 associates with several repair and recombination proteins such as RAD51 (16), RAD50/MRE11/NBS1 (17) (18), and MSH2/MSH6 (18). BRCA1 also interacts with and is phosphorylated by protein kinases that are key players in the damage checkpoint control, including ATM, ATR, and CHK2 (19) (20) (21). Lastly, it has been shown recently that BRCA1 preferentially binds to branched DNA structures (22). Despite these findings, the exact mechanism that BRCA1 uses to facilitate the DNA repair process remains to be understood.

In addition to its potential role in DNA repair, BRCA1 has also been implicated in regulation of transcription (7) (23). When tethered to a transcriptional promoter via a heterologous DNA binding domain, the C-terminal 304-amino-acid region including the BRCT repeats (aa 1560-1863; AD2 in Fig. 1B) can act as a *trans*-activation domain (24) (25). More recent work has revealed a second *trans*-activation domain of BRCA1 that resides upstream of the BRCT repeats (26) (aa1293-1559; AD1 in Fig. 1B). The two activation domains (AD1 and AD2) can cooperatively activate transcription in many cell lines tested (26). Consistent with its potential role in transcriptional regulation, the BRCA1 polypeptide is associated with the RNA polymerase II holoenzyme via RNA helicase A (RHA) (27) (28). Furthermore, BRCA1 interacts with a number of site-specific transcription factors and modulates their actions in gene activation (29) (30) (31) (32) (33) (34) (35) (36) (37) (38).

The multifunctional nature of BRCA1 has raised the possibility that the protein may employ a common mechanism, such as chromatin remodeling, to regulate various chromosomal events. Indeed, the C-terminal region of BRCA1 (AD2), which is required for BRCA1 functions in both DNA repair and transcription {Scully, 2000 #65}{Monteiro, 2000 #67}, can induce changes in nucleosome structure when tethered to chromosomal DNA in *Saccharomyces cerevisiae* (39). Furthermore, BRCA1 is associated with histone modifying enzymes (p300 and HDAC) (28) (40) (41) and an ATP-dependent chromatin remodeling machine (hSNF/SWI) (42). The fact that many cancer-predisposing mutations reduce BRCA1's affinity for these chromatin-modifying proteins suggests that chromatin remodeling may be an important aspect of BRCA1-mediated tumor suppression. However, currently there is no direct evidence in mammalian cells for BRCA1-mediated changes in chromatin structure. This is in part due to the lack of convenient assays for directly monitoring chromatin remodeling at different levels of chromatin structure in mammalian cells.

The overall objectives of this grant proposal are to use yeast genetic approaches to identify potential cofactors of BRCA1 that mediate its role in chromatin reorganization and regulation of multiple nuclear functions. As described below, we have accomplished the original research goals by successfully isolating both novel and known mammalian proteins that act as functional cofactors

of BRCA1. These data provide strong evidence for a functional link between chromatin reorganization and BRCA1 function in tumor suppression.

BODY OF THE REPORT

Characterization of the BRCA1 BRCT domain and comparative study of BRCT-containing proteins The BRCA1 C-terminal (BRCT) domain is present in a number of proteins that are involved in various aspects of chromosomal events (43) (44). When tethered to chromosomal DNA, this region of BRCA1 is capable of inducing changes in chromatin structure (39). Despite the sequence homology and functional proximity shared by the BRCT-containing proteins, it is not clear whether different BRCT domains confer a common biochemical activity. Much less is known about the functional significance of the characteristic amino acid residues in the BRCT motif. In the first year of the funding period, we conducted a functional comparison between the BRCT domain of BRCA1 and those of several *Saccharomyces cerevisiae* proteins with known nuclear functions. Our work shows that chromatin remodeling and transcription activation is not a common feature of BRCT domains. Nevertheless, the BRCT domain of the multi-functional repressor-activator protein 1 (RAP1) is capable of activating transcription and remodeling chromatin in a manner similar to that shown for the BRCT domain of BRCA1. Mutational analysis demonstrates that most of the conserved amino acid residues in the BRCA1 BRCT domain are essential for its function in transcriptional activation. In contrast, mutations of many analogous amino acid residues in the RAP1 BRCT domain greatly elevate the transcriptional activity. These data indicate that the conserved residues in these two BRCT domains may play different roles in transcriptional activation. This work was published in *J. Biol. Chem.* (45).

Isolating a novel BRCA1-interacting protein through yeast genetic screens The multifunctional nature of BRCA1 has raised the possibility that the polypeptide may regulate various nuclear processes via a common underlying mechanism such as chromatin remodeling. However, to date no direct evidence exists in mammalian cells for BRCA1-mediated changes in either local- or large-scale chromatin structure. Here we show that targeting BRCA1 to a specific chromosome location in the mammalian genome results in large-scale chromatin decondensation. This unfolding activity is independently conferred by three subdomains within the *trans*-activation domain (AD) of BRCA1, namely, activation domain 1 (AD1) and the two BRCA1 C-terminus (BRCT) repeats. In addition, we also demonstrate a similar chromatin unfolding activity associated with the *trans*-activation domains of E2F1 and tumor suppressor p53. However, unlike E2F1 and p53, the BRCT-mediated chromatin unfolding is not accompanied by histone hyperacetylation. Cancer-predisposing mutations of *BRCA1* display an allele-specific effect on chromatin unfolding: 5' mutations that result in gross truncation of the protein abolish the chromatin unfolding activity, whereas those in the 3' region of the gene markedly enhance this activity. A novel cofactor of BRCA1 (COBRA1) is recruited to the chromosome site by the first BRCT repeat of BRCA1 and is itself sufficient to induce chromatin unfolding. BRCA1 mutations that enhance chromatin unfolding also increase its affinity for, and recruitment of, COBRA1. These results indicate that reorganization of higher levels of chromatin structure is an important regulated step in BRCA1-mediated nuclear functions. This work was published in *J. Cell Biol.* (46).

BRCA1 and JunB interact with each other and act in concert to modulate gene expression in breast and ovarian cells. BRCA1 has been implicated in regulation of multiple nuclear events including transcription. AD1, one of the two *trans*-activation domains in BRCA1, stimulates transcription in a cell-context dependent manner. We recently show that a coiled-coil motif in AD1, which is critical for its function in transcriptional activation, interacts with the basic leucine zipper (bZIP) region of the Jun proteins. In particular, the cellular level of JunB correlates with the activation potency of AD1. Ectopic expression of JunB, but not that of c-Jun, JunD, or c-Fos, potentiates the transcriptional activity of AD1. Thus, the coiled-coil-mediated cooperation between BRCA1 and JunB may facilitate the function of these proteins in

transcriptional regulation and tumor suppression. This work was recently published in *J. Biol. Chem.* and *Genes & Dev.* (26) (47).

KEY RESEARCH ACCOMPLISHMENTS

1) Identification of a subset of BRCT domains that are capable of remodeling chromatin, activating transcription and DNA replication.

2) Identification of a novel protein that binds to BRCA1 and facilitate its function in chromatin remodeling.

3) Establishment of a functional link between BRCA1 and JunB in transcriptional regulation and suppression of ovarian cancer development.

REPORTABLE OUTCOMES

Miyake, T., Hu, Y-F., Yu, D.S., and Li, R. (2000) A functional comparison of BRCA1 C-terminal domains in transcription activation and chromatin remodeling. *J. Biol. Chem.* 275, 40169-40173.

Hu, Y-F., Miyake, T., Ye, Q., and Li, R. (2000) Characterization of a novel *trans*-activation domain of BRCA1 that functions in concert with the BRCA1 C-terminal (BRCT) domain. *J. Biol. Chem.* 275, 40910-40915.

Ye, Y., Hu, Y-H., Belmont, A., and Li, R. (2001) BRCA1-mediated high-order chromatin unfolding and its deregulation by cancer predisposing mutations. *J. Cell Biol.*, 155:911-921.

Hu, Y-F. and Li, R. (2002) JunB Potentiates function of BRCA1 Activation Domain 1(AD1) through a coiled-coil-mediated interaction. Genes & Dev. 16:1509-1517.

CONCLUSIONS

One focus of the research goals in this proposal is to characterize the BRCT domain for its function in chromatin remodeling and regulation of multiple nuclear events including transcription and DNA replication. We have achieved this part of the research goals by determining the critical amino acid residues responsible for the BRCT activity (45). We also compared the activity and amino acid requirement of the BRCA1 BRCT domains with other BRCT-containing proteins (45). A second major goal in this proposal is to use yeast genetic tools to identify BRCA1-interacting proteins. We have accomplished this goal by isolating COBRA1 and JunB as the functional and physical partners of BRCA1 (26) (47) (46). Therefore, the work represents an important contribution to the understanding of the molecular basis of BRCA1-mediated familial breast cancer.

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APPENDICES

Reprints of four publications as listed in REPORTABLE OUTCOMES.

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A Functional Comparison of BRCA1 C-terminal Domains in Transcription Activation and Chromatin Remodeling*

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The BRCA1 C-terminal (BRCT) domain is present in a number of proteins that are involved in various aspects of chromosomal events. The BRCT domain of BRCA1 is important for its function in DNA repair and transcriptional activation. When tethered to chromosomal DNA, this region of BRCA1 is capable of inducing changes in chromatin structure. Despite the sequence homology and functional proximity shared by the BRCT-containing proteins, it is not clear whether different BRCT domains confer a common biochemical activity. Much less is known about the functional significance of the characteristic amino acid residues in the BRCT motif. Here, we show that chromatin remodeling and transcription activation is not a common feature of BRCT domains. However, the BRCT domain of the multi-functional repressor-activator protein 1 (RAP1) can activate transcription and remodel chromatin in a manner similar to that shown for the BRCA1 BRCT domain. Most of the conserved amino acid residues in the second BRCA1 BRCT domain are essential for its function in transcriptional activation. In contrast, mutations of many analogous amino acid residues in the RAP1 BRCT domain greatly elevate the transcriptional activity. These data indicate that the conserved residues in these two BRCT domains may play different roles in transcriptional activation.

Mutations of *BRCA1* account for a large proportion of familial breast and ovarian cancers (1, 2). Multiple lines of evidence suggest that BRCA1 is involved in regulation of several nuclear functions, including transcription, DNA repair, recombination, and checkpoint control (3–11). The entire 1863 amino acid protein contains a highly conserved RING finger domain at the N terminus and two repeats of the BRCA1 C-terminal (BRCT)[1] domain at the C terminus. Although most cancer-predisposing mutations of *BRCA1* result in gross truncation of the protein, 5–10% of the disease-associated mutations lead to single amino acid substitutions, many of which occur in the RING or BRCT domains, suggesting that both domains are pivotal to BRCA1 function in tumor suppression.

The BRCT domain of BRCA1 is required for its function in both transcriptional activation and DNA repair (3–10). When tethered via the GAL4 DNA binding domain (DBD), the BRCT domain is capable of stimulating transcription from a GAL4-responsive reporter gene (3, 4) and remodeling chromatin from chromosome-embedded GAL4 binding sites (13). The same region is also reported to interact with histone-modifying enzymes such as the histone acetyltransferase p300 (14, 15) and the human histone deacetylase, HDAC (16). Importantly, cancer-predisposing mutations in this region abolish the ability of BRCA1 to activate transcription, enhance DNA repair efficiency, and remodel chromatin. Thus, BRCA1 may utilize the BRCT domain to increase chromatin accessibility and facilitate multiple chromosomal events.

The BRCA1 BRCT domain also shares a limited sequence homology with a large number of proteins that are involved in various aspects of chromosomal events, such as DNA repair, replication, recombination, gene activation and silencing, and checkpoint control (17, 18). The functional proximity of the BRCT superfamily opens up the possibility that BRCT domains may share a common activity in regulation of nuclear functions. Several recent reports suggest a role of BRCT domains in mediating protein-protein interactions with each other or with a different structural module. For example, the BRCT domains present in DNA ligase III and XRCC1, two mammalian DNA repair proteins, interact strongly with each other (19). In addition, the BRCT domain of BRCA1 binds CtIP, a transcriptional co-repressor (20–22). Despite these findings, it remains to be determined whether different BRCT domains utilize a common biochemical feature to regulate various nuclear processes.

The BRCT motif is an approximately 100-amino acid long region defined by distinct conserved patches of hydrophobic residues (17, 18) (also see the alignment in Fig. 3). An x-ray crystallographic study of the second BRCT domain of the repair protein XRCC1 shows that it contains a four-stranded parallel β-sheet encircled by three α-helices (23). Based on the XRCC1 BRCT structure, a model for the BRCT domain of BRCA1 has been constructed, and a few cancer-predisposing mutations in this region are predicted to either disrupt the interface or cause incorrect folding (23). However, most of the characteristic amino acid residues in the BRCA1 BRCT domain have not been associated with familial breast or ovarian cancers. Therefore, the relevance of these conserved residues to BRCA1 function remains to be explored.

In this study, we compared the potentials of the BRCT domains from BRCA1 and several yeast proteins in transcription activation and chromatin remodeling. Our work shows that most BRCT domains, with the exception of the scRAP1 BRCT domain, do not display *in vivo* properties similar to those shown for the BRCA1 BRCT domain. Mutational study of the BRCA1 and scRAP1 BRCT domains also indicates that many of the characteristic amino acid residues of the BRCT motif play

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[1] The abbreviations used are: BRCT, BRCA1 C-terminal; MNase, micrococcal nuclease; HA, hemagglutinin; DBD, DNA binding domain; ARS, autonomously replicating sequence; RAP1, repressor-activator protein 1; aa, amino acid; h, human; sc, *Saccharomyces cerevisiae*.

distinct roles in transcriptional activation by these BRCT domains. Taken together, our findings point to the functional diversity and structural complexity among the BRCT superfamily members.

EXPERIMENTAL PROCEDURES

Plasmids and Cells—For the yeast-based transcription assay shown in Fig. 1*C*, the *lacZ* reporter plasmid carrying two GAL4 binding sites was integrated into the yeast strain RL1 as described previously (24). To construct the reporter plasmid with five GAL4 binding sites used in Fig. 5*B*, an *Xba*I-*Hin*dIII fragment containing the five GAL4 sites from the vector G5BCAT (25) was blunt-ended and cloned into the blunt-ended *Xho*I site in the *lacZ* reporter construct pJL638 (26). The resulting plasmid was linearized with *Stu*I and integrated at the *URA3* locus. The luciferase reporter plasmid used in human cells contains four GAL4 binding sites in front of the *fos* TATA element as described previously (27).

Site-directed mutagenesis was performed using the Kunkel method as described (28). To construct the yeast expression vectors for the GAL4 fusion proteins, the sequences encoding the following BRCT domains were amplified by a standard polymerase chain reaction method: BRCA1 (aa 1560–1863); DPB11 (Fig. 1*A*, fragments *a* and *b*; aa 1–220); DPB11 (fragments *c* and *d*; aa 322–579); ESC4 (fragments *e* and *f*; aa 841–1070); RAD9 (aa 1027–1309); RAP1 (aa 121–208); and RFC1 (aa 153–243). The polymerase chain reaction fragments were subsequently cloned into the *Xba*I and *Bam*HI sites immediately downstream of the HA-GAL4(1–94) sequence in the *CUP1* expression vector described previously (24). The entire sequence of the amplified fragments was verified by sequencing. The resulting expression vectors were integrated into the *LEU2* locus on the chromosome. For expression of the fusion proteins in human cells, the BRCT domain of BRCA1 was cloned into the *Xba*I-*Bam*HI sites in the expression vector pCG-GAL4(1–94)-HA (27).

The RL1 yeast strain used for the micrococcal nuclease (MNase) sensitivity assay and the β-galactosidase assay was described previously (24). Human HEK293T cells, generously provided by T. Ouchi at the Mount Sinai School of Medicine, were maintained in Dulbecco's modified Eagle's medium with 10% fetal calf serum.

Immunoblotting and MNase Assay—These assays were performed as described previously (24), except that all liquid growth media contained 100 μM copper sulfate for induction of the GAL4 derivatives. The immunoblots were probed with an anti-HA antibody (12CA5).

Transcription Assays—The yeast transcription assay was performed and the specific activity calculated following a standard protocol (29). For the luciferase assay, human HEK293T cells were transfected using LipofectAMINE 2000 from Life Technologies, Inc. The following plasmids were included in the transfection: the luciferase reporter (0.5 μg), the β-galactosidase reporter (0.5 μg), and the appropriate GAL4 fusion expression plasmid (1 μg). Transfected cells were harvested 40 h later, and the luciferase and β-galactosidase activities were determined following the procedures previously described (27). The β-galactosidase activity was used as an internal control for transfection efficiency.

RESULTS AND DISCUSSION

Transcription Activation Is Not a Universal Feature of BRCT Domains—To test the possibility that BRCT domains besides that of BRCA1 may also be capable of activating transcription, we fused the hemagglutinin (HA)-tagged GAL4-DBD to the BRCT domains of several proteins from *Saccharomyces cerevisiae* (Fig. 1*A*). The BRCT-containing proteins chosen here are known for their roles in the regulation of various aspects of chromosomal events. These proteins include Dpb11p, involved in DNA replication and S-phase checkpoint (30); Rfc1p, in DNA replication and repair (31); Rad9p, in DNA damage checkpoint (32); Rap1p, in transcriptional activation, silencing, and telomere length maintenance (33, 34); and Esc4p, in gene silencing (17). As shown in Fig. 1*B*, all GAL4 fusion proteins were expressed with the expected sizes and at a comparable level in yeast.

The potential of these fusion proteins to activate transcription in yeast was analyzed using a GAL4-responsive *lacZ* reporter gene (Fig. 1*C*). Consistent with previous findings (3, 4), GAL4-BRCA1 activated transcription in yeast as well as human cells (compare columns *1* and *2* in Fig. *1C*; also see Fig. 4).

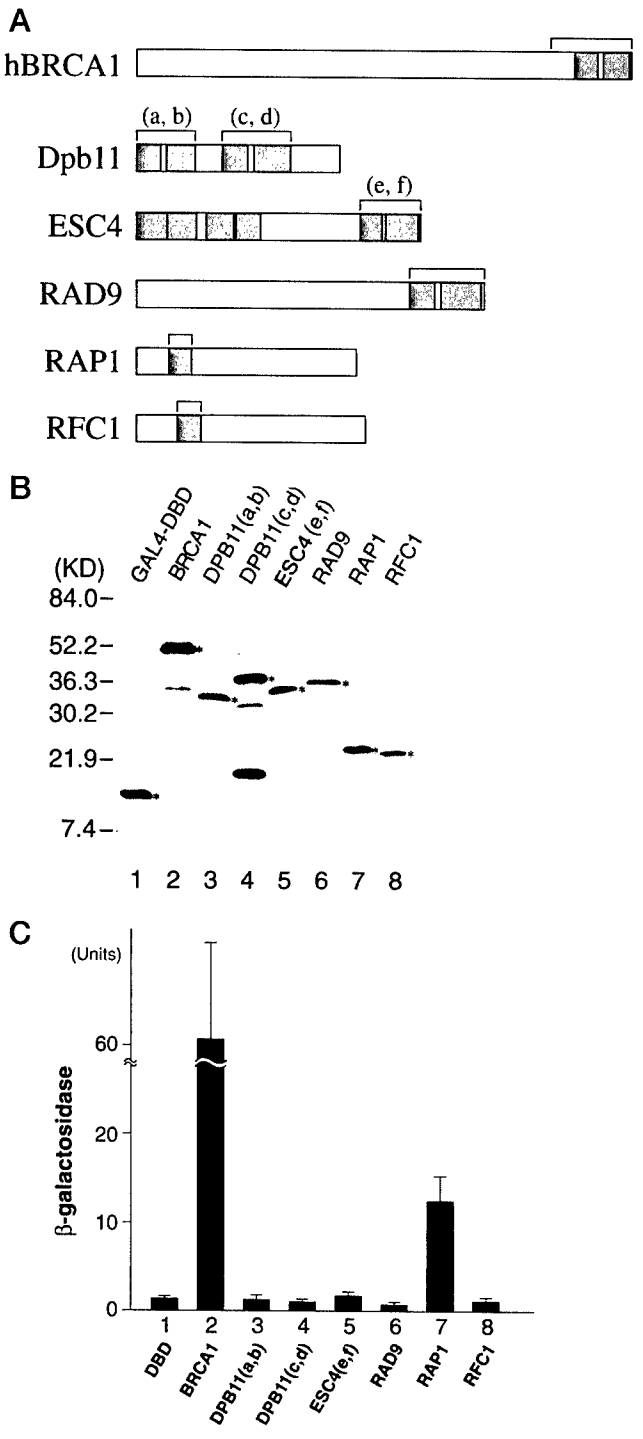

FIG. 1. **Comparison of BRCT domains in transcriptional activation.** *A*, diagram illustrating the relative position of the BRCT domains in BRCA1 and the yeast proteins. Each *shaded* region represents one BRCT motif. The regions marked by *brackets* are those included in the same GAL4 fusion protein. *B*, the HA-tagged GAL4 derivatives were detected by immunoblotting of an equal amount of whole cell extracts. The molecular mass (in kD) markers are indicated on the *left*. *Asterisks* indicate the bands representing the full-length fusion proteins. See the text for more description of each fusion protein. *C*, β-galactosidase activity from cells that express various GAL4-BRCT fusion proteins. The results shown are an average of data from at least four independent experiments.

Of all the other BRCT fusion proteins tested, only GAL4-RAP1 gave rise to an elevated β-galactosidase activity (compare columns *1* with *7*). None of the remaining GAL4-BRCT constructs showed any appreciable levels of transcriptional stimulation (columns *3–6* and *8*). Although it remains possible that the lack

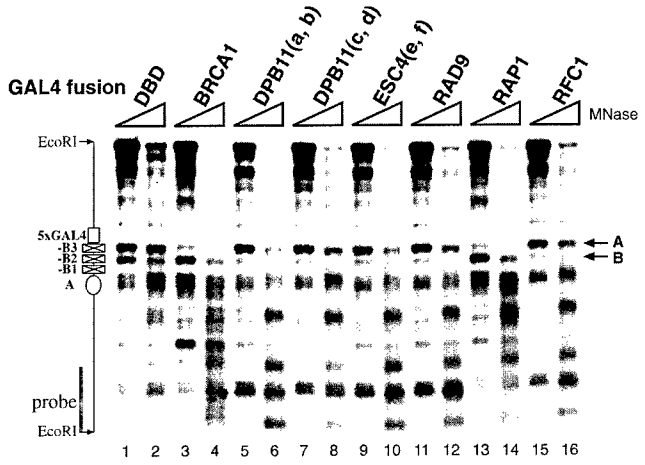

FIG. 2. **The BRCT domains from BRCA1 and scRAP1 can induce changes of the chromatin structure *in vivo*.** Indirect end-labeling assay was used to reveal the MNase digestion pattern around the chromosome-embedded GAL4 binding sites. Two time points of the MNase treatment are shown for each GAL4 derivative. The *Eco*RI fragment containing the *ARS1* locus was revealed by a radioactive probe that corresponds to one end of the restriction fragment (indicated by a *thick bar* on the *left*). The *arrows* (labeled *A* and *B*) indicate the two bands in which the intensities were most significantly affected by GAL4-BRCA1 and GAL4-RAP1. The approximate positions of the GAL4 binding sites and the four *cis*-elements of *ARS1* are indicated on the *left*.

of transcription activation may be because of improper folding of the BRCT domains in the GAL4 fusion context, our data suggest that only a subset of the BRCT domains shares a common activity in transcriptional activation.

The hBRCA1 and scRAP1 BRCT Domains Can Remodel Chromatin—Next, we examined the potentials of the GAL4 derivatives to induce changes in chromatin structure. The yeast strain used in this study contains five GAL4 binding sites engineered near the autonomously replicating sequence 1 (ARS1) on chromosome IV (24). It has been shown that, although all GAL4 derivatives can get access to the chromosome-embedded GAL4 binding sites, only those that activate transcription and chromosomal replication induce distinct changes in the MNase digestion pattern around the GAL4 binding sites (13). As shown in Fig. 2, tethering of the BRCT domain of BRCA1 to the chromosome resulted in chromatin remodeling, namely, the intensity of band A was attenuated, whereas that of band B was enhanced (indicated by *arrows*; compare *lanes 1* and *2* with *3* and *4* in Fig. 2). The same changes were observed with GAL4-RAP1 (*lanes 13* and *14*) but not with any of the transcriptionally inactive GAL4-BRCT fusion proteins (*lanes 5–12* and *15–16*). Thus, transcriptional activation by the BRCT domains correlates with their ability to induce changes in chromatin structure. Like transcriptional activation, chromatin remodeling is shared by only a subset of the BRCT domains.

The observed changes in chromatin structure are unlikely to be caused by the presence of the entire transcription machinery or initiation of transcription *per se* in the vicinity of the GAL4 sites, as the GAL4 binding sites are located at least 800 base pairs away from the nearest transcriptional promoter. Rather, a chromatin remodeling complex may be recruited by the hBRCA1 and scRAP1 BRCT domains to induce the changes in the local chromatin structure. In keeping with this notion, the BRCT domain of BRCA1 has been shown to associate with certain histone modifying enzymes such as the histone acetyltransferase p300 (14, 15) and the human histone deacetylase, HDAC (16). Taken together, the transcription activation and chromatin remodeling assays may provide useful tools for identifying additional BRCT domains that share the same biochemical features as those of scRAP1 and BRCA1.

Most of the Conserved Residues in the BRCA1 BRCT Domain Are Critical for Transcriptional Activation—To address the significance of the characteristic amino acid residues of the BRCT motif in transcriptional activation, we introduced mutations in several conserved blocks of the second BRCT domain of BRCA1 (*Hs BRCA1-b* in Fig. 3). These include the N-terminal tail, α1, α3, and the C-terminal tail as predicted based on the structure of the XRCC1 BRCT domain (23). The four-stranded β-sheet and α2 were not targeted for mutagenesis in this study, as the β-sheet is predicted to form the core of the structure and the corresponding mutant proteins tend to be unstable (data not shown), whereas the sequence for α2 is not universally present in the BRCT superfamily.

A total of 11 alanine substitution mutants were constructed in the context of the GAL4-BRCA1 fusion. To analyze the mutational effects in a more physiological context, the wild-type and mutant fusion proteins were expressed and their transcriptional activity assessed in human HEK293T cells (Fig. 4, *A* and *B*). As expected, the wild-type GAL4-BRCA1 fusion can potently stimulate transcription from a GAL4-responsive luciferase reporter construct (compare *lane 2* with *lane 1* in Fig. 4*B*). Mutations at multiple conserved amino acid residues either reduced or completely abolished the transcriptional activity. For example, I1760A/F1761A, L1780A, M1783A/V1784A, G1788A, V1838A/L1839A, S1841A, and Y1853A/L1854A/I1855A all severely impaired the *trans*-activation capability of the BRCT domain (compare *lane 2* with *lanes 3, 6, 8–11,* and *13* in Fig. 4*B*). Of all the conserved residues mutated in this study, only L1764A failed to affect BRCT function in a significant manner (*lane 4* in Fig. 4B). In addition, mutations of three nonconserved residues (D1778A, W1782A, and Q1848A) did not cause deleterious effects either (Fig. 4*B*, *lanes 5, 7,* and *12*), despite the fact that the analogous residues of Asp^{1778} and Trp^{1782} in XRCC1 are involved in dimer formation between two BRCT domains (23). The behaviors of the mutant proteins are summarized below the sequence of the BRCT domain in Fig. 3.

Natural mutations at Met^{1783}, Gly^{1788}, and Ser^{1841} have been identified as unclassified mutant variants in terms of cancer predisposition and predicted to affect protein folding (23). The alanine substitution mutants at these three positions indeed abrogated the transcriptional activation by GAL4-BRCA1 (*lanes 8, 9,* and *11* in Fig. 4*B*), suggesting that the corresponding natural mutations at these sites may represent genuine cancer-predisposing mutations. Taken together, the data strongly suggest that a majority of the conserved amino acid residues in the BRCT domain are critical for BRCA1 function in transcriptional activation. Our results in human cells are also in agreement with a recent yeast-based study of the BRCA1 BRCT domain (35). In particular, the three regions mutagenized in both studies (Phe^{1761}, Leu^{1780}, and Tyr^{1853}-Leu^{1854}-Ile^{1855}) are important for *trans*-activation in human as well as yeast cells.

Distinct Roles of the Conserved Residues in the scRAP1 BRCT Domain—Given the sequence homology and functional similarity between the BRCA1 and scRAP1 BRCT domains, we asked whether the analogous residues in the scRAP1 BRCT domain are required for transcriptional activation as well. Site-directed mutagenesis was employed to change the amino acid residues analogous to those in the BRCA1 BRCT domain studied above. As illustrated in Fig. 3, alanine substitutions were introduced at Pro^{125}-Leu^{126} of the N terminus; Leu^{143}, Asn^{144}, Leu^{149}, Arg^{151}, and Leu^{152}-Ile^{153} of α1; Gly^{157}-Gly^{158} between α1 and β2; Tyr^{190}, Ile^{191}-Lys^{192}, and Cys^{194} of α3; and Tyr^{206}-Leu^{207}-Val^{208} of the C-terminal tail.

The potentials of the mutant GAL4-RAP1 fusion proteins in

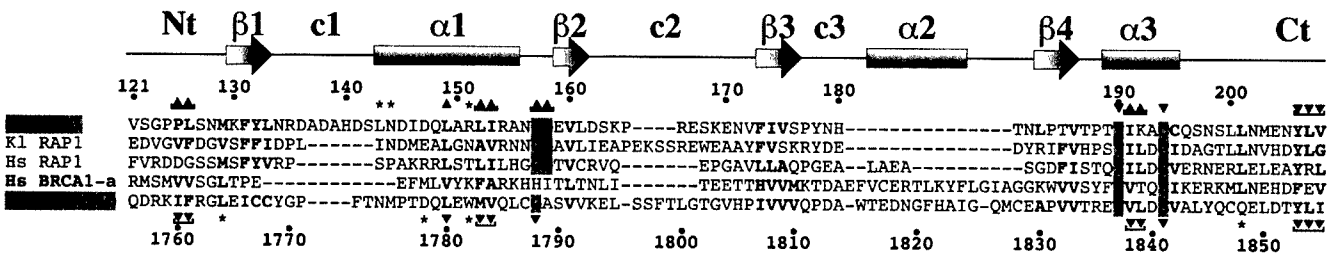

FIG. 3. **Diagram showing the primary sequence and the predicted secondary structure of the BRCT domains.** Alignment of BRCT domains of three RAP1 proteins is done using ClustalW and structural information (38). Sc, *Saccharomyces cerevisiae*; Kl, *Kluyveromyces lactis*; Hs, *Homo sapiens*. The most invariant amino acids in the BRCT domains are indicated by *bold type* and *shading*, and the conserved hydrophobic residues are in *bold type* only. The *numbers above* and *below* the sequences correspond to the positions of amino acids in scRAP1 and hBRCA1-b, respectively. *Asterisks* and *triangles* indicate those amino acids that are mutated in this study. *Asterisks* are those that show no obvious phenotypes. The *upward triangles* designate mutants that cause "super-activation," and the *downward triangles* indicate mutations that reduce transcriptional activity.

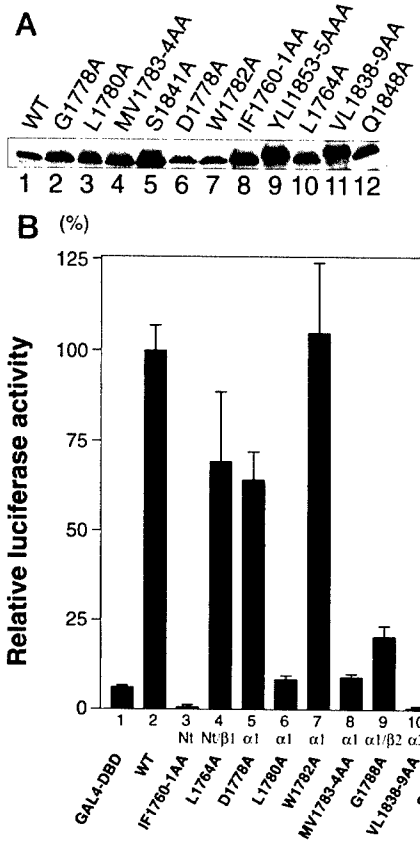

FIG. 4. **Mutational analysis of the second BRCT domain of BRCA1 in human cells.** *A*, expression of the HA-tagged GAL4-BRCA1 chimeras in human HEK293T cells. The immunoblot is probed with an anti-HA antibody, 12CA5. *B*, luciferase assays are performed using cell lysates from human HEK293T cells transfected with a luciferase reporter, a β-galactosidase reporter (as an internal control), and the GAL4-BRCA1 expression vectors. The transcriptional activity for the wild-type (*WT*) construct is set at 100%. Indicated at the *bottom* of the graph are the mutations and the predicted secondary structures where the mutations are located. *Nt*, N-terminal tail; *Ct*, C-terminal tail.

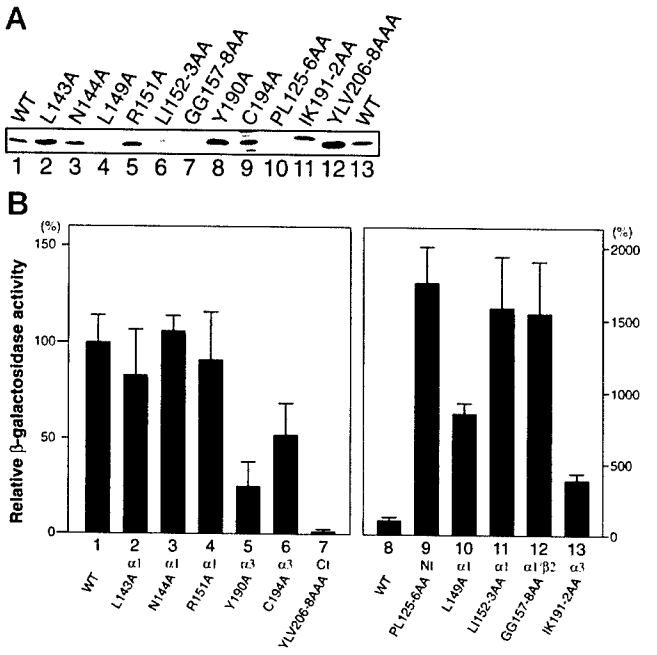

FIG. 5. **Mutational analysis of the scRAP1 BRCT domain in *S. cerevisiae*.** *A*, immunoblot showing the expression of various GAL4-RAP1 fusion proteins in yeast cells. *B*, liquid β-galactosidase assays are carried out using lysates from yeast cells that express various GAL4 derivatives. For a convenient comparison, the β-galactosidase values are shown in two different scales (*left* and *right panels*). The wild-type (*WT*) activity is set at 100%. Indicated at the *bottom* of the graph are the mutations and the predicted secondary structures where the mutations are located.

transcription activation were analyzed in a yeast β-galactosidase assay. As shown in Fig. 5*B*, two mutations in α3 (Y190A and C194A) and one in the C-terminal tail (Y206A/L207A/V208A) either reduced or completely abolished the ability of GAL4-RAP1 to stimulate transcription (Fig. 5*B*, *lanes 5–7*). These mutants were expressed at a similar level as the wild-type protein (compare *lanes 1, 8, 9,* and *12* in Fig. 5*A*). Notably, the corresponding residues in the BRCA1 BRCT domain had a similar deleterious effect on transcriptional activation (see W1837R in Ref. 13 and S1841A and Y1853A/L1854A/I1855A in Fig. 4*B*). Furthermore, both W1837R and a nonsense mutation that results in deletion of Tyr[1853]-Leu[1854]-Ile[1855] in BRCA1 are found to be associated with familial breast cancer. Therefore, the conserved residues toward the C terminus of the BRCT motif are required for both BRCA1 and scRAP1 BRCT domains to stimulate transcription.

In contrast to the three mutations mentioned above, changes at many other conserved positions in the scRAP1 BRCT domain greatly increased the transcriptional activity of the fusion protein (compare *lane 8* with *lanes 9–13* in Fig. 5*B*; also note the two scales for *lanes 1–7* and *8–13*). Most strikingly, P125A/L126A, L152A/I153A, and G157A/G158A were at least 15-fold as robust as the wild-type protein (compare *lane 8* with *lanes 9, 11,* and *12* in Fig. 5*B*). In addition, L149A and I191A/K192A also resulted in a significant increase in β-galactosidase activity, albeit to a lesser extent (*lanes 10* and *13*). Interestingly, some of these "superactivating" mutants were expressed at lower levels than the wild-type GAL4-RAP1 (*lanes 4, 6, 7,* and *10* in Fig. 5*A*). Therefore, as summarized in Fig. 3, although the

C termini of the BRCA1 and scRAP1 BRCT domains are required for both domains to activate transcription, many of the characteristic amino acid residues of the BRCT motif appear to play distinct roles in these two BRCT domains. This finding further supports the notion that the signature residues of the BRCT motif are not sufficient to confer a common function among the BRCT superfamily members.

Based on the crystal structure of the XRCC1 BRCT domain (23), the residues involved in repressing the BRCT function in scRAP1 (Pro^{125}-Leu^{126}, Leu^{149}, Leu^{152}-Ile^{153}, Gly^{157}-Gly^{158}, and Ile^{191}-Lys^{192}) are predicted to reside proximally in the tertiary structure. Moreover, Leu^{149} and Leu^{152}-Ile^{153} of $\alpha 1$ and Ile^{191}-Lys^{192} of $\alpha 3$ are likely to be involved in mediating the intramolecular interaction between $\alpha 1$ and $\alpha 3$, whereas the highly conserved Gly-Gly residues between $\alpha 1$ and $\beta 2$ may be important for proper orientation of the two helices. Thus, it is conceivable that the interaction between $\alpha 1$ and $\alpha 3$ may result in a conformation that is unfavorable for transcriptional activation by the RAP1 BRCT domain. Alternatively, the amino acid residues of interest may be involved in an interaction with a transcriptional repressor. In either situation, the negative regulatory region of the scRAP1 BRCT domain may serve as a molecular switch that coordinates the multiple functions of RAP1 in transcriptional activation, gene silencing (33), and telomere length maintenance (36), etc. However, it remains formally possible that the scRAP1 BRCT domain may fold in a conformation distinct from that of the BRCA1 and XRCC1 BRCT domains.

The scRAP1 BRCT domain is identified by sequence comparison, yet its role in the biological function of the full-length protein remains obscure. In fact, the entire N-terminal sequence including the BRCT domain is not essential for scRAP1 to support cell viability (37). In this regard, it is somewhat puzzling that the BRCT domain is well conserved between the yeast and human RAP1 proteins, whereas the sequence for the potent *trans*-activation domain at the C terminus of scRAP1 is not present as such in hRAP1 (38). Although the exact contribution of the BRCT domain to RAP1 function awaits further investigation, it is possible that the transcriptional activation and chromatin remodeling property associated with the BRCT domain may be important for a more specialized, nonessential function of scRAP1. It is also tempting to speculate that, in the absence of a strong *trans*-activation domain in hRAP1, the BRCT domain may play a more prominent role in RAP1-mediated regulation of chromosomal events in human cells.

In conclusion, the current work demonstrates that most of the characteristic amino acid residues of the BRCA1 BRCT domain are required for its function in transcription activation. However, these conserved residues are not sufficient to confer a similar *in vivo* activity on other members of the BRCT superfamily. The study also shows that the evolutionarily conserved BRCT domain in RAP1 is capable of transcription activation and chromatin remodeling. However, unlike the BRCA1 BRCT domain, many of the characteristic residues in the RAP1 BRCT domain are involved in negative regulation of transcription activation. Thus, there appears to be a significant degree of structural complexity and functional diversity among different BRCT domains.

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Characterization of a Novel *Trans*-Activation Domain of BRCA1 That Functions in Concert with the BRCA1 C-terminal (BRCT) Domain*

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Mutations in the breast cancer susceptibility gene, *BRCA1*, account for a significant proportion of hereditary breast and ovarian cancers. The BRCA1 C-terminal (BRCT) domain, which can activate transcription when fused to a heterologous DNA binding domain, is required for BRCA1 function in suppression of tumorigenesis. Here, we provide evidence for a new activation domain in BRCA1 that lies adjacent to the BRCT domain. We name the two domains AD1 and AD2, respectively. Like AD2, the newly discovered AD1 can act independently as an activation domain in both yeast and human cells. However, unlike AD2, AD1 activity in mammalian cells is cell type context-dependent. Furthermore, combination of these two domains in mammalian cells can result in a robust synergy in transcriptional activation. A highly conserved coiled-coil motif in AD1 is required for the cooperative transcription activation. Interestingly, the functional cooperativity between AD1 and AD2 is absent in certain breast and ovarian cancer cell lines, although each domain can still activate transcription. Therefore, the differential and cooperative actions of the two activation modules may contribute to the heterogeneous risk of BRCA1 mutations in different tissues.

Between 5 and 10% of all breast cancers are hereditary, and most of these are caused by germline mutations in two breast cancer susceptibility genes, *BRCA1* and *BRCA2* (1, 2). The remaining 90–95% of breast cancers are classified as sporadic. The human *BRCA1* gene encodes a 1863-amino acid protein with a highly conserved RING finger domain at the N terminus and two repeats of the BRCT[1] domain at the C terminus (see Fig. 1*A*). Although most cancer-predisposing mutations of *BRCA1* result in gross truncation of the protein, 5–10% of the disease-associated mutations lead to single amino acid substitutions (5). Many of the cancer-predisposing single-point mutations occur in the RING or BRCT domains, underscoring the importance of these two domains to BRCA1 function in tumor suppression.

The exact biochemical function of the BRCA1 protein has been the focus of intense research. Several lines of evidence suggest that BRCA1 is involved in DNA repair (6–9). Embryonic stem cells from BRCA1-deficient mice are hypersensitive to ionizing radiation, presumably because of defects in transcription-coupled repair of oxidative DNA damage as well as double-strand break-induced homologous recombination (6, 8, 10). The role of BRCA1 in DNA repair is further supported by the observation that it associates with several well known repair and recombination proteins such as RAD51 (11), RAD50/MRE11/NBS1 (12, 13), and MSH2/MSH6 (13). BRCA1 also interacts with and is phosphorylated by ATM and CHK2 (7, 14), two protein kinases that are key players in damage checkpoint control.

It has also been suggested that BRCA1 is involved in regulation of transcription. When tethered to a transcriptional promoter via a heterologous DNA binding domain, the C-terminal 304-amino acid region (aa 1560–1863) including the BRCT domain can act as a *trans*-activation domain (3, 4). The same C-terminal region of BRCA1 can remodel chromatin when tethered to chromosomal DNA (15). Consistent with its potential role in transcriptional regulation, the BRCA1 polypeptide is associated with the RNA polymerase II holoenzyme via RNA helicase A (16, 17). It has also been reported that, when overexpressed in mammalian cells, the full-length BRCA1 protein can potentiate transcription from several natural promoters in both a p53-dependent and -independent manner (18–21). Finally, BRCA1 is associated with histone-modifying enzymes (p300 and histone deacetylase) (17, 21, 22) and an ATP-dependent chromatin remodeling machine (hSNF/SWI) (23). Thus, BRCA1 may utilize the BRCT domain to increase chromatin accessibility and facilitate multiple nuclear processes.

During the course of further characterization of BRCA1-mediated transcriptional activation, we discovered a novel *trans*-activation domain of BRCA1 that resides immediately upstream of the BRCT domain. We designated this new and previously known domain as AD1 and AD2, respectively. Although both domains can act as *trans*-activation domains, AD1 activity is restricted by cellular contexts to a greater extent than AD2 activity. Furthermore, the two activation domains can cooperatively activate transcription in many cell lines tested. A highly conserved coiled-coil region in BRCA1 is critical for the functional synergy between these two activation domains. Thus, our findings imply that other cellular and molecular modifiers could influence the biochemical property of BRCA1.

EXPERIMENTAL PROCEDURES

Plasmids and Cell Lines—The mammalian luciferase reporter plasmid was described previously (24). To construct the mammalian expression vectors for the GAL4 fusion proteins, the sequences encoding

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[1] The abbreviations used are: BRCT, BRCA1 C-terminal; HA, hemagglutinin; AD, activation domain; NLS, nuclear localization signal; aa, amino acid(s); PCR, polymerase chain reaction; CTF1, CCAAT-box binding transcription factor 1; ATM, ataxia telangiectasia mutated.

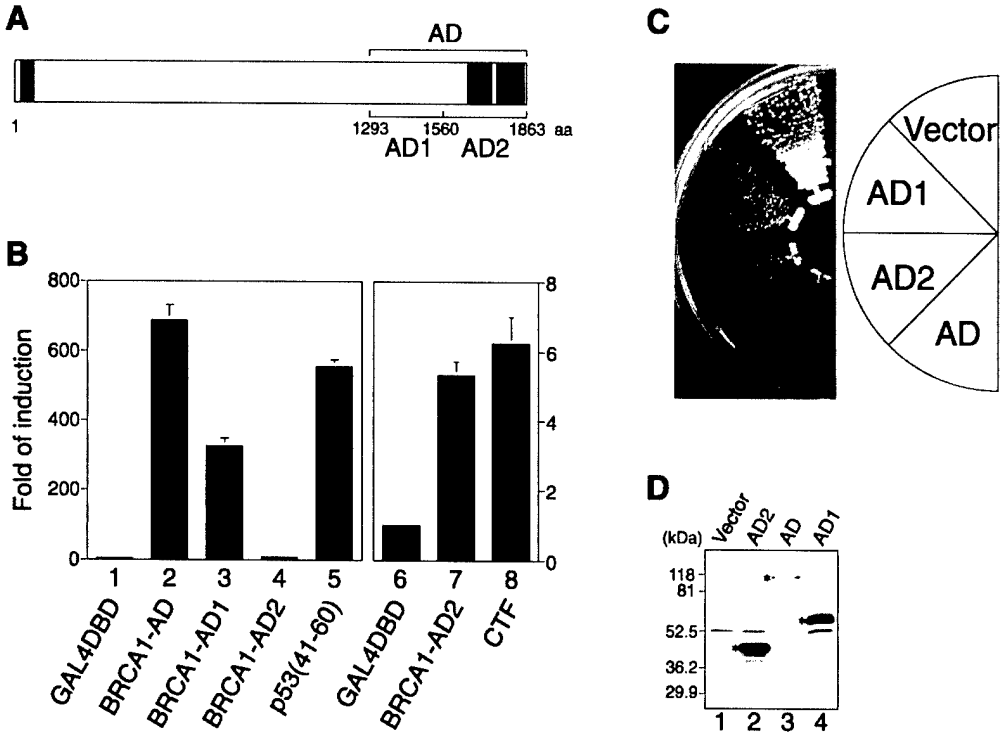

FIG. 1. **The effect of AD1 on transcriptional activation and growth inhibition in yeast.** *A*, diagram illustrating the boundaries of AD1, AD2, and AD in BRCA1. The *solid regions* at the C terminus represent the two BRCT motifs. The *solid region* at the N terminus is the RING domain. *B*, activation of the LacZ expression in HF7c cells that express various GAL4-BRCA1 fusion proteins. The results shown are an average of data from at least four independent experiments. The stimulatory effects of AD1 and the activation domain of CTF1 (aa 399–499) are presented on a different scale in *columns 6–8*. *C*, SP1 cells with various galactose-inducible expression vectors were streaked on synthetic complete medium without uracil but with 5% galactose and 1% raffinose. The plate was photographed 4 days after incubation at 30 °C. *D*, detection of the HA-NLS-BRCA1 proteins. SP1 cells with the appropriate expression vectors were grown for 24 h in galactose-containing liquid medium. Cell lysates were prepared, and the induced proteins were detected by immunoblotting with an anti-HA antibody, 12CA5. An equal amount of lysate was loaded in each *lane*.

various lengths of the BRCA1 C-terminal region were amplified from a BRCA1 cDNA clone by a standard PCR method. The PCR fragments were subsequently cloned into the *Nhe*I and *Bam*HI sites in the mammalian expression vector pCG-GAL4-(1–94)-HA (24). Point mutations were introduced by the site-directed mutagenesis method as described previously (25).

Human HEK293T cells and HeLa cells were grown in DMEM with 10% fetal bovine serum. HCT116, a generous gift of B. Vogelstein at the Johns Hopkins Oncology Center, was maintained in McCoy5a with 10% fetal bovine serum. The remaining cell lines used in the study were purchased from the American Tissue Culture Center and cultured as instructed by the vendor.

For expression of the GAL4 fusion proteins in yeast, the corresponding BRCA1 sequences were cloned into the *Nhe*I and *Xho*I sites of the two-hybrid bait plasmid pGBT8 (CLONTECH). The expression vectors used in the yeast growth inhibition assay were constructed as follows. First, an oligonucleotide encoding the nuclear localization signal (NLS) from simian virus 40 (SV40) T-antigen was inserted at the *Xba*I site between the sequences for the HA tag and BRCA1 in pCG-GAL4-HA-BRCA1 (aa 1560–1863) (37). The sequence for HA-NLS-BRCA1(aa 1560–1863) was subsequently amplified by PCR. The amplified fragment, which contained an initiation codon in front of the sequence for the HA tag, was cloned between the *Xba*I and *Bam*HI sites in p416GAL1 (26). The resulting plasmid, pTM268, encodes the fusion protein HA-NLS-BRCA1 (aa 1560–1863) under the control of the *GAL1* promoter. To over-express similar fusion proteins with different C-terminal regions of BRCA1 (AD, aa 1293–1863; AD1, aa 1293–1558), the BRCA1 sequence in pTM268 was replaced with the corresponding DNA fragments from the vector pcDNA3β(HA)BRCA1 (16). The yeast strains HF7c (CLONTECH) and SP1 (27) were used for the yeast β-galactosidase assay and growth inhibition experiment, respectively.

Immunoblotting—The assay was performed as described previously (28). 12CA5, a monoclonal antibody raised against the HA epitope, was used in all immunoblots.

Transcription Assays—The yeast transcription assay was performed, and the specific activity was calculated following a standard protocol (29). For the luciferase assay, human cells were transfected using the following methods: LipofectAMINE 2000 (Life Technologies, Inc.) for HEK293T and SKOV-3, electroporation (Bio-Rad) for HCC1937, and LipofectAMINE Plus (Life Technologies, Inc.) for the rest of the cell lines. The following plasmids were included in each transfection: the luciferase reporter (0.5 μg), the β-galactosidase reporter (0.5 μg), and the appropriate GAL4 fusion expression plasmid (1 μg). Transfected cells were harvested 40 h later, and luciferase and β-galactosidase activities were determined following the procedures described previously (24). β-Galactosidase activity was used as an internal control for transfection efficiency.

RESULTS

Transcriptional Activation in Yeast by the BRCA1 C-Terminal Domains—Previous work has shown that, when tethered to the appropriate chromosomal regions in *Saccharomyces cerevisiae*, the C-terminal 304 amino acids of BRCA1 (aa 1560–1863) including the BRCT repeats can activate transcription (4), stimulate DNA replication (15), and remodel chromatin (15). In addition, over-expression of the same region results in growth inhibition in yeast (30). Importantly, these yeast-based activities are abrogated by cancer-predisposing mutations in the BRCT domain, suggesting that the yeast system may serve as a powerful genetic tool for dissecting BRCA1 function.

During the course of further exploration of the validity of the yeast-based approach, we observed that the originally defined activation domain exhibited only a modest activity in transcription activation (Fig. 1*B*; compare *column 1* with *4* and *column 6* with *7* at a different scale). As a comparison, its activity was equivalent to that of the CTF1 activation domain, a relatively weak mammalian activation domain (compare *column 7* with *8*, Fig. 1*B*). However, inclusion of an additional 267-aa region immediately upstream of the BRCT domain elicited a much greater level of activation (compare *column 2* with *4*, Fig. 1*B*).

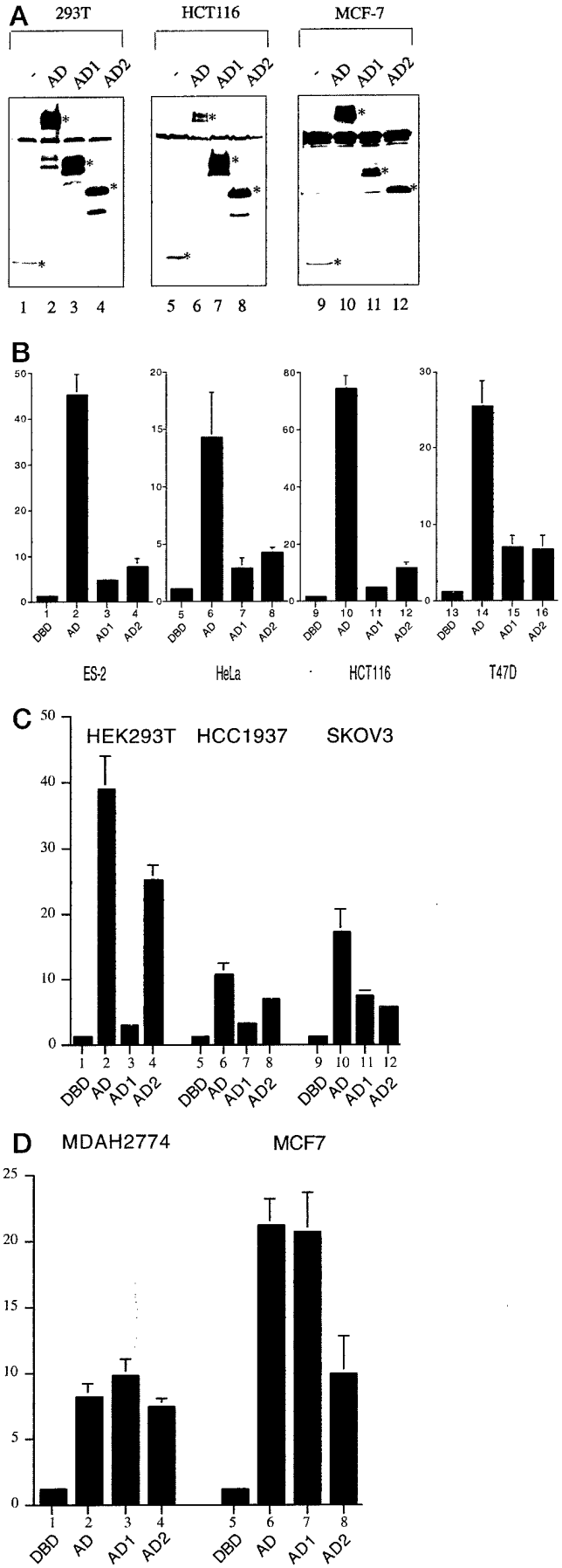

FIG. 2. **Activation of transcription in mammalian cells by GAL4-BRCA1 fusion proteins.** *A*, immunoblots showing expression of GAL4-DBD-HA alone (*lanes 1*, *5*, and *9*) and GAL4-DBD-HA fused with AD, AD1, and AD2. An anti-HA antibody, 12CA5, was used. The three different cell lines shown here represent the three categories discussed in the text. *Asterisks* indicate the positions of the expected full-length proteins. *B*, the first category of cell lines, which supports a synergistic activation by AD1 and AD2. *DBD*, DNA-binding domain. The *y* axis shows the fold of activation. *C*, the second category, which shows an additive effect by the joint action of AD1 and AD2. *D*, the third category, in which AD1 and AD2 cannot exert a concerted effect on gene expression. All results represent at least three independent experiments. In some columns the S.D. is too small to show up.

Furthermore, this 267-aa upstream sequence alone acted as a more potent activation domain than the BRCT domain (compare *column 3* with *4*). In fact, the new region was almost as active as a strong acidic activation domain from tumor suppressor p53 (compare *column 3* with *5*, Fig. 1*B*). These results indicate that the region between aa 1293 and 1559 of BRCA1 may contain a previously unidentified independent *trans*-activation domain. In the following experiments, we refer to this 267-aa region and the original activation domain as activation domain 1 and 2 (AD1, aa 1293–1558; AD2, aa 1560–1863), respectively, and the entire 571-aa C-terminal region of BRCA1 as the activation domain (aa 1293–1863; *AD*, Fig. 1*A*).

Next, we asked whether the newly identified AD1 would influence AD2-mediated growth inhibition in yeast. BRCA1 sequences encoding AD1, AD2, or AD (AD1 + AD2) were fused with the hemagglutinin (HA) epitope and a NLS from the SV40 large T antigen. Expression of the fusion genes was under the control of a galactose-inducible promoter, *GAL1*. Consistent with previous findings (30), over-expression of the BRCT domain (AD2) impaired yeast cell growth (Fig. 1*C*). A moderate inhibitory effect was observed with the over-expressed AD1 fusion protein as well. However, expression of AD, which contains both AD1 and AD2, imposed a more pronounced growth inhibition than expression of either AD1 or AD2 alone (Fig. 1*C*), despite the fact that HA-NLS-AD was expressed less abundantly than the two smaller fusion proteins (Fig. 1*D*). Interestingly, it has been reported that expression of a BRCA1 fragment that includes both AD1 and AD2 in mammalian cells also causes a severe abnormality in cell cycle control (31). Taken together, these results strongly suggest that both AD1 and AD2 in the C terminus of BRCA1 are required for its maximal function in transcription activation.

Comparison of Different BRCA1 Activation Domains in Mammalian Cells—To confirm the yeast results in a more physiologically relevant context, we carried out a similar study using a mammalian transcription assay. Given the tissue-specific nature of the BRCA1-dependent neoplasm, we examined the transcription potentials of AD, AD1, and AD2 in human cancer cell lines of various origins, including breast (MCF7, T47D, and HCC1937), ovary (MDAH2774, SKOV3, and ES2), colon (HCT116), cervix (HeLa), and kidney (HEK293T). All three HA-tagged GAL4 derivatives were expressed at a comparable level in each of the cell lines tested (Fig. 2*A* and data not shown).

Based upon their ability to support a collaborative activation by AD1 and AD2, the cell lines tested in this study can be divided into three categories. In the first category, AD1 and AD2 can synergistically activate transcription (Fig. 2*B*). The most striking synergism was observed in HCT116, a colorectal carcinoma cell line. As shown in *columns 9–12* of Fig. 2*B*, the GAL4 chimeras with either AD1 or AD2 alone only resulted in a less than 10-fold activation, whereas conjunction of the two domains (AD) yielded a robust 75-fold activation. Notably, GAL4-AD was expressed at a lower level than the GAL4 chimera with either AD1 or AD2 alone (*lanes 6–8* in Fig. 2*A*).

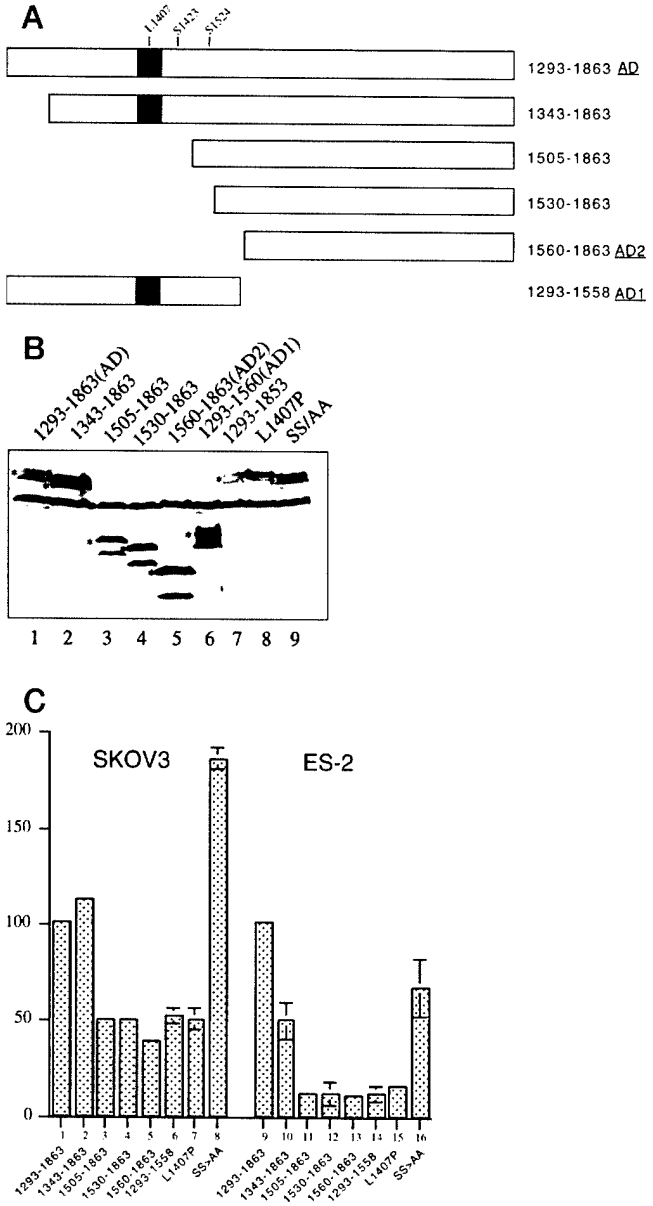

FIG. 3. **Mutational analysis of AD1 function in transcriptional activation.** *A*, diagram showing the relative positions of various deletional and point mutants analyzed in the transcription assays. The coiled-coil region is *highlighted* in *black*. The point mutations are shown at the *top*. *B*, immunoblot showing expression of the HA-tagged GAL4 fusion proteins. An anti-HA antibody, 12CA5, was used. *Asterisks* indicate the positions of the expected full-length fusion proteins. *C*, the effect of various mutations on the ability of AD to activate transcription. The wild-type GAL4-AD activity is set as 100.

Thus, the stronger activation by GAL4-AD could not be attributed to the difference in the levels of the activators. A similar cooperativity was also found in three other cell lines derived from ovarian (ES-2), cervical (HeLa), and breast (T47D) cancers (Fig. 2*B*, *columns 1–8* and *13–16*).

The second category, which includes HEK293T (kidney), HCC1937 (breast), and SKOV3 (ovary), supports a less concerted activation by AD1 and AD2 than the first category (Fig. 2*C*). For example, AD1 and AD2 together only resulted in an additive effect on transcription activation in SKOV3 cells (*columns 9–12*, Fig. 2*C*). In the other two cell lines of the same category, HEK293T and HCC1937, AD1 alone did not display an appreciable activity (*columns 3* and *7*, Fig. 3*C*). However, AD1 contributed moderately to the activity of AD (compare *columns 2* and *4* and *6* and *8* in Fig. 3*C*). Of all the cell lines tested in our study, HEK293T cells displayed the most dramatic difference between AD1 and AD2 activities (*columns 3* and *4*, Fig. 2*C*), despite the fact that GAL4-AD1 was more abundant than GAL4-AD2 in these cells (*lanes 3* and *4* in Fig. 2*A*).

The last category, which includes an ovarian (MDAH2774) and a breast (MCF7) cancer cell line, fails to support a concurrent activation by AD1 and AD2 (Fig. 2*D*). For instance, AD1 and AD2 can individually activate transcription in MDAH2774 cells (*columns 3* and *4*, Fig. 2*D*). However, a combination of the two modules in MDAH2774 cells resulted in the same degree of activation as did each domain alone (*column 2*, Fig. 2*D*). The three GAL4-derived activators were expressed at a comparable level in these two cell lines (*lanes 10–12* in Fig. 2*A* and data not shown).

A Coiled-coil Region in AD1 Is Important for Transcription Activation—To identify the sequence determinants critical for AD1 function in transcription activation, a series of N-terminal deletion mutants were constructed in the context of the GAL4-AD fusion protein (Fig. 3*A*) and analyzed in both ES-2 and SKOV3 cells. All fusion proteins were expressed at similar levels in each cell line (Fig. 3*B* and data not shown). As described above, the presence of both AD1 and AD2 resulted in a synergistic effect in ES-2 cells (*columns 9, 13*, and *14*, Fig. 3*C*) and an additive effect in SKOV3 cells (*columns 1, 5*, and *6*). Deletion of the sequence between aa 1343 and 1505 essentially abolished the synergistic effect in ES-2 (*column 11*, Fig. 3*C*) as well as the additive effect in SKOV3 cells (*column 3*). Further truncations from the N terminus of AD did not significantly affect the residual transcription activity (*e.g.* compare *column 3* with *4* and *5*, Fig. 3*C*).

Using several secondary structure assignment programs, we identified a coiled-coil motif in the region, as shown by the deletional study, to be critical for AD1 function. This coiled-coil motif is located between aa 1391 and 1424 and is the only such sequence in the entire BRCA1 protein (Fig. 4). As indicated in Fig. 4*A*, it contains a series of leucines or other hydrophobic residues at positions *a* and *d* of the α-helical wheel. The amino acids in the coiled-coil motif, in particular the hydrophobic residues, are evolutionarily conserved among BRCA1 homologues of different species (Fig. 4*A*). To assess the relevance of the coiled-coil motif to AD1 function, we mutated the highly conserved leucine residue in the middle of the coiled-coil motif (L1407P). Based upon previous findings on other coiled-coil proteins, the leucine-to-proline change is known to disrupt the coiled-coil structure (32). As shown in *columns 7* and *15* of Fig. 3*C*, the L1407P mutation indeed abrogated both the additive effect of the two domains in SKOV3 cells and the synergistic effect in ES-2 cells. The same mutation also severely impaired AD1 function when it acted alone as an activation domain (data not shown). These data strongly suggest that the coiled-coil motif is important for BRCA1 function in transcription activation.

In addition to the coiled-coil motif, the AD1 sequence also contains a number of serine and threonine residues followed by glutamine residues (SQ or TQ). In particular, phosphorylation at Ser^{1423} and Ser^{1524} by the ATM kinase is important for BRCA1 function in DNA damage response (7). Because the two serine residues are located either within or near the region shown to be important for AD1 activity, we mutagenized both serine residues. As shown in Fig. 3*C*, the double mutant (SS → AA) did not cause any deleterious effects on AD activity (compare *column 1* with *8*, and *9* with *16*). However, this finding does not exclude a possible role for these residues in transcription activation by BRCA1 following DNA damage.

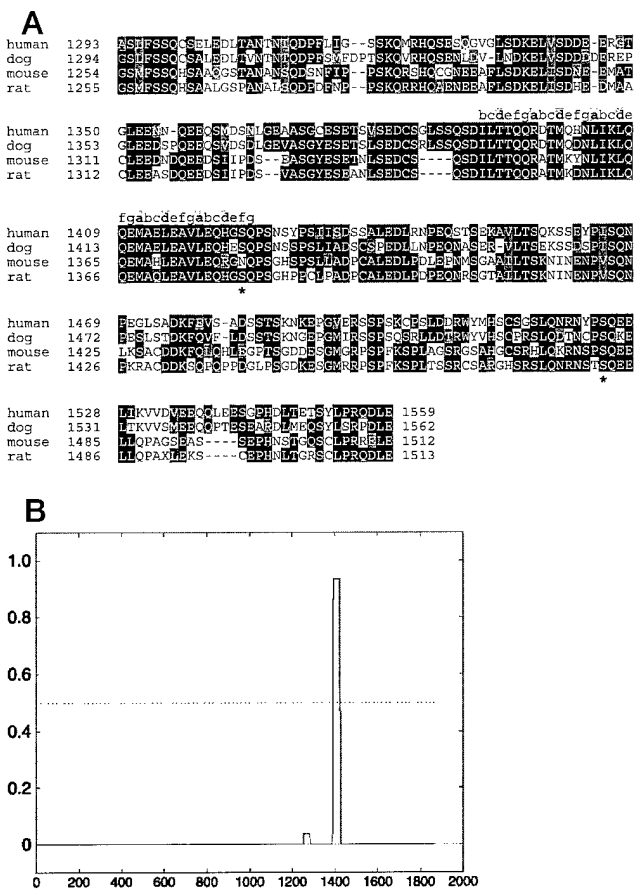

FIG. 4. **Structural features of the C-terminal region of BRCA1.** *A*, sequence alignment of the BRCA1 C-terminal region from human, mouse, rat, and dog. The conserved amino acid residues are *highlighted* in *black*, and similar residues are *shaded*. The *letters a–g* on *top* of the coiled-coil sequence (aa 1391–1424) indicate amino acid residues of the heptad repeats. The hydrophobic *a* and *d* residues, which are the critical determinants for the coiled-coil motif, are *highlighted*. The *asterisks* at Ser^{1423} and Ser^{1524} indicate the ATM phosphorylation sites. *B*, prediction of the coiled-coil structure in BRCA1. Coiled-coil structure was predicted by the PAIRCOIL program (35). The *x* axis indicates the amino acid residue *numbers* of BRCA1, and the *y* axis indicates the probability of a coiled-coil motif. The probability cutoff (0.5) is shown as a *dashed line*. A similar result was obtained using a different secondary structure prediction program (Ref. 36; data not shown).

DISCUSSION

Although it has been well accepted that the C terminus of BRCA1 is required for its function in transcription activation and DNA repair, the boundary and complexity of the activation domain remain to be thoroughly examined. Moreover, compared with other known *trans*-activation domains, the originally identified activation domain possesses only a modest transcriptional activity *in vivo* (Ref. 22 and this study; see Fig. 1*B*). Our work in yeast and human cells has led to the discovery of a new activation domain (AD1) in BRCA1 that can function coorperatively with the BRCT domain (AD2). This study also reveals a putative coiled-coil domain that is critical for the role of AD1 in transcription activation. Finally, the cell type-dependent behavior of the activation domains implies the existence of other genetic modifiers that can modulate the transcriptional function of BRCA1.

Although both AD1 and AD2 are capable of independently stimulating transcription, they behave differently under various cellular contexts. For example, GAL4-AD1 activity in HEK293T cells was marginal, yet GAL4-AD2 expressed in the same cell line gave rise to transcription stimulation as much as 25-fold (Fig. 2*C*). In contrast, both activation domains were equally active in MDAH2774 (Fig. 2*D*). In general, AD1 activity tends to be more dependent upon the cellular environment, whereas AD2 activates transcription in a ubiquitous manner, which could explain why AD1 was not identified as an independent activation domain in earlier functional studies.

Gayther *et al.* (33) report that a higher likelihood of ovarian (*versus* breast) cancer correlates with a *BRCA1* mutation closer to the 5′ terminal region of the gene. Rather than a gradual transition through the entire gene, the change in ovarian cancer risk occurs at a sharp demarcation point between aa 1435 and 1443. Based on this observation, it is postulated that a domain near the transition point may bind to a tissue-specific factor(s), which in turn confers a specific protection against familial ovarian cancer. Intriguingly, the transition point immediately follows the coiled-coil motif of AD1 that is defined in our study (aa 1391–1424). Given that coiled-coil motifs are known for their function in mediating protein-protein interactions (32), the coiled-coil region in AD1 is an excellent candidate for the proposed protective domain. BRCA1 mutations that leave this domain unaffected may result in lower risks of ovarian cancer, whereas truncating mutations that abolish the interaction between the coiled-coil motif and its putative target protein may substantially increase the risk of developing ovarian cancer.

The molecular basis for the different behaviors of the cell lines remains to be elucidated. The concerted activation by AD1 and AD2 observed in the first two categories has been well documented for other eukaryotic transcription activators; this is likely due to the concurrent interactions of these two domains with their corresponding target proteins in the basal transcription machinery and/or chromatin-modifying machines. However, the failure to support a joint action of the two activation domains, as shown in Fig. 2*D*, is quite puzzling. The relative abundance of the target proteins of AD1 and AD2 cannot easily account for the deficiency, as each domain can individually activate transcription in the same cell lines. The phenomenon is probably not due to the status of the endogenous BRCA1 either, as the protein is expressed at a comparable level in all the cell lines tested except for HCC1937,[2] a BRCA1-deficient breast cell line in which a truncated form of BRCA1 is expressed at a lower level (34). To explain the apparent lack of a concerted action of AD1 and AD2, we speculate that an additional factor may be required to integrate the stimulatory effect of the two activation domains. It is possible that lack of such a coordinating factor may prevent a concerted action of AD1 and AD2. Alternatively, the compositions of the target proteins for AD1 and AD2 in these cell lines are such that the two activation domains may not be able to simultaneously recruit their corresponding targets to the same promoter.

Sporadic forms of breast and ovarian cancers are far more common than the hereditary types. However, disease-associated somatic mutations in BRCA1 have rarely been described in the sporadic tumors. Thus, it is largely unknown whether BRCA1 is involved in sporadic tumorigenesis. Previous studies have suggested that BRCA1 may play a role in sporadic cancer development through mechanisms other than mutations in its coding region, such as regulation of expression, differential splicing, and altered cellular localization (for a review, see Ref. 2). Our finding that certain breast and ovarian cancer cell lines fail to support a concerted activation by AD1 and AD2 may provide yet another possible mechanism by which malfunction of the BRCA1-mediated transcriptional activation may contribute to the development of sporadic breast and ovarian cancers. However, this apparently would explain only a subset of spo-

[2] Q. Ye, unpublished data.

radic forms of cancer, as not all breast and ovarian cancer cell lines in our study lose their ability to support a concerted action of AD1 and AD2. Nevertheless, given the heterogeneous clinical features of both breast and ovarian cancers and the multiple nuclear events in which BRCA1 has been implicated, it is entirely possible that development of these cancers may be triggered by dysfunction in different aspects of the BRCA1-mediated processes.

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Article

BRCA1-induced large-scale chromatin unfolding and allele-specific effects of cancer-predisposing mutations

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The breast cancer susceptibility gene *BRCA1* encodes a protein that has been implicated in multiple nuclear functions, including transcription and DNA repair. The multifunctional nature of BRCA1 has raised the possibility that the polypeptide may regulate various nuclear processes via a common underlying mechanism such as chromatin remodeling. However, to date, no direct evidence exists in mammalian cells for BRCA1-mediated changes in either local or large-scale chromatin structure. Here we show that targeting BRCA1 to an amplified, lac operator–containing chromosome region in the mammalian genome results in large-scale chromatin decondensation. This unfolding activity is independently conferred by three subdomains within the transactivation domain of BRCA1, namely activation domain 1, and the two BRCA1 COOH terminus (BRCT) repeats. In addition, we demonstrate a similar chromatin unfolding activity associated with the transactivation domains of E2F1 and tumor suppressor p53. However, unlike E2F1 and p53, BRCT-mediated chromatin unfolding is not accompanied by histone hyperacetylation. Cancer-predisposing mutations of *BRCA1* display an allele-specific effect on chromatin unfolding: 5′ mutations that result in gross truncation of the protein abolish the chromatin unfolding activity, whereas those in the 3′ region of the gene markedly enhance this activity. A novel cofactor of BRCA1 (COBRA1) is recruited to the chromosome site by the first BRCT repeat of BRCA1, and is itself sufficient to induce chromatin unfolding. BRCA1 mutations that enhance chromatin unfolding also increase its affinity for, and recruitment of, COBRA1. These results indicate that reorganization of higher levels of chromatin structure is an important regulated step in BRCA1-mediated nuclear functions.

Introduction

Germ line mutations in breast cancer susceptibility gene 1 (*BRCA1*)* confer elevated risks in the development of familial breast and ovarian cancers (Rahman and Stratton, 1998). *BRCA1* encodes a 1,863–amino acid protein with a highly conserved ring finger motif (RING) at the NH_2 terminus, and two BRCA1 COOH terminus (BRCT) repeats at the extreme COOH terminus. Whereas most disease-associated mutations of BRCA1 are predicted to result in gross truncation of the protein, 5–10% of the cancer-predisposing mutations cause single amino acid substitutions, many of which are located in the RING domain or BRCT repeats.

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*Abbreviations used in this paper: aa, amino acid(s); AAD, acidic activation domain; BRCA1, breast cancer susceptibility gene 1; BRCT, BRCA1 COOH terminus; COBRA1, cofactor of BRCA1; EGFP, enhanced green fluorescent protein; GST, glutathione *S*-transferase; RING, ring finger motif.

Key words: BRCA1; BRCT; chromatin unfolding; breast cancer; COBRA1

Intense research in the past several years has implicated BRCA1 in the regulation of multiple nuclear processes, including DNA repair and transcription (Zhang et al., 1998b; Scully and Livingston, 2000). For example, BRCA1-deficient mouse and human cells are hypersensitive to ionizing radiation due to defects in transcription-coupled repair of oxidative DNA damage, as well as double-strand break-induced homologous recombination (Gowen et al., 1998; Abbott et al., 1999; Moynahan et al., 1999; Scully et al., 1999; Xu et al., 1999). In addition, BRCA1 associates with several repair and recombination proteins such as RAD51 (Scully et al., 1997b), RAD50/MRE11/NBS1 (Zhong et al., 1999; Wang et al., 2000), and MSH2/MSH6 (Wang et al., 2000). BRCA1 also interacts with and is phosphorylated by protein kinases that are key players in the damage checkpoint control, including ATM, ATR, and CHK2 (Cortez et al., 1999; Lee et al., 2000; Tibbetts et al., 2000). Lastly, it has been shown recently that BRCA1 preferentially binds to branched DNA structures (Paull et al., 2001).

In addition to its potential role in DNA repair, BRCA1 has also been implicated in regulation of transcription

(Monteiro, 2000; Scully and Livingston, 2000). When tethered to a transcriptional promoter via a heterologous DNA binding domain, the COOH-terminal 304–amino acid (aa) region including the BRCT repeats (Fig. 1 B, AD2, amino acids 1560–1863) can act as a transactivation domain (Chapman and Verma, 1996; Monteiro et al., 1996). More recent work has revealed a second transactivation domain of BRCA1 that resides upstream of the BRCT repeats (Hu et al., 2000) (Fig. 1 B, AD1, aa 1293–1559). The two activation domains (ADs), AD1 and AD2, can cooperatively activate transcription in many cell lines tested (Hu et al., 2000). Consistent with its potential role in transcriptional regulation, the BRCA1 polypeptide is associated with the RNA polymerase II holoenzyme via RNA helicase A (Scully et al., 1997a; Neish et al., 1998). Furthermore, BRCA1 interacts with a number of site-specific transcription factors and modulates their actions in gene activation (Somasundaram et al., 1997; Ouchi et al., 1998, 2000; Zhang et al., 1998a; Fan et al., 1999; Houvras et al., 2000; Zheng et al., 2000).

The multifunctional nature of BRCA1 has raised the possibility that the protein may employ a common mechanism, such as chromatin remodeling, to regulate various chromosomal events. Indeed, the COOH-terminal region of BRCA1 (AD2), which is required for BRCA1 functions in both DNA repair and transcription (Monteiro, 2000; Scully and Livingston, 2000), can induce changes in nucleosome structure when tethered to chromosomal DNA in *Saccharomyces cerevisiae* (Hu et al., 1999). Furthermore, BRCA1 is associated with histone modifying enzymes (p300 and HDAC) (Neish et al., 1998; Yarden and Brody, 1999; Pao et al., 2000) and an ATP-dependent chromatin remodeling machine (hSNF/SWI) (Bochar et al., 2000). The fact that many cancer-predisposing mutations reduce BRCA1's affinity for these chromatin-modifying proteins suggests that chromatin remodeling may be an important aspect of BRCA1-mediated tumor suppression. However, currently there is no direct evidence in mammalian cells for BRCA1-mediated changes in chromatin structure. This is in part due to the lack of convenient assays for directly monitoring chromatin remodeling at different levels of chromatin structure in mammalian cells.

A lac repressor–based system has allowed direct visualization of large-scale chromatin dynamics in mammalian cells (Belmont, 2001). In this system, multiple copies of the lac operator were engineered into the genome of CHO cells, and together with the surrounding genomic sequences, were amplified to produce a 90-Mb heterochromatic region. By fusing lac repressor with the acidic AD (AAD) of the strong viral transcription factor VP16 and tethering the fusion protein to the heterochromatic chromosome region, this system was used to demonstrate AAD-induced large-scale chromatin decondensation (Tumbar et al., 1999). This large-scale chromatin uncoiling occurred even when RNA pol II–dependent transcription was blocked, suggesting that it was induced through transacting factors recruited by the VP16 AAD, rather than the result of transcription per se. Conceptually, the transacting factors producing this higher order chromatin decondensation could be one of the known chromatin-modifying complexes that modify local nucleosome structure (Peterson and Logie, 2000). Alternatively, AAD-induced chromatin unfolding could involve novel factors acting primarily at the higher levels of chromatin organization.

Although artificial, this lac repressor–tethering system provides a very quick, and therefore powerful, assay to test the possible role of specific proteins in chromatin remodeling and to dissect the protein domains required for the observed large-scale chromatin decondensation. Using this lac repressor–tethering assay, we demonstrate here that BRCA1 induces large-scale chromatin decondensation. We also identify three small subdomains within the transactivation domain of BRCA1 that are capable of independently conferring chromatin unfolding. In addition, cancer-predisposing mutations of BRCA1 display allele-specific effects on the chromatin unfolding activity. Finally, we isolate a novel cofactor of BRCA1 (COBRA1) that binds to one of the chromatin-unfolding domains of BRCA1, and by itself induces large-scale chromatin decondensation. Our results suggest that BRCA1-mediated decondensation of higher levels of chromatin structure may represent a new physiological regulatory pathway related to BRCA1 function. The approach used in the current study also provides a new methodology for identifying novel BRCA1-interacting proteins involved in this regulatory pathway.

Results

BRCA1-mediated large-scale chromatin decondensation in mammalian cells

To assess the impact of BRCA1 on large-scale chromatin structure in mammalian cells, we made use of a CHO cell line, AO3_1, in which multiple copies of the lac operator were engineered to produce a 90-Mb heterochromatic region of the genome (Robinett et al., 1996; Li et al., 1998; Tumbar et al., 1999). The molecular organization of this region consists of ~400-kb repeats of the 14-kb vector transgene that contains the lac operator repeat and dihydrofolate reductase selectable marker. The repeats are separated on average by ~1,000 kb of unknown coamplified genomic DNA. Because other cell clones derived from the same selection procedure contain more open, gene-amplified chromosome regions with comparable or greater content of the vector DNA, the heterochromatic appearance of the A03_1 chromosome region is assumed to be due to properties of the coamplified genomic DNA. In vivo binding of lac repressor or its GFP derivatives to this chromosomal site allows direct visualization of large-scale chromatin dynamics without altering the original chromosome structure.

Consistent with previous findings, lac repressor–expressing cells stained with the corresponding antibody exhibited a compact nuclear dot (Fig. 1 A, a). In contrast, expression of lac repressor fused with the full-length BRCA1 induced an irregularly shaped subnuclear structure in 14% of transfected cells (Fig. 1 A, b). Such a staining pattern was not present in any of the cells expressing lac repressor alone. These results suggest that BRCA1, or a BRCA1-associated protein, can induce large-scale chromatin restructuring. The magnitude of this opening was lower than observed for the VP16 AAD, and was present in a lower percentage of cells (14 vs. 60% for VP16 AAD) (Tumbar et al., 1999 and see Fig. 2). The lack of a response in 100% of cells, even for the

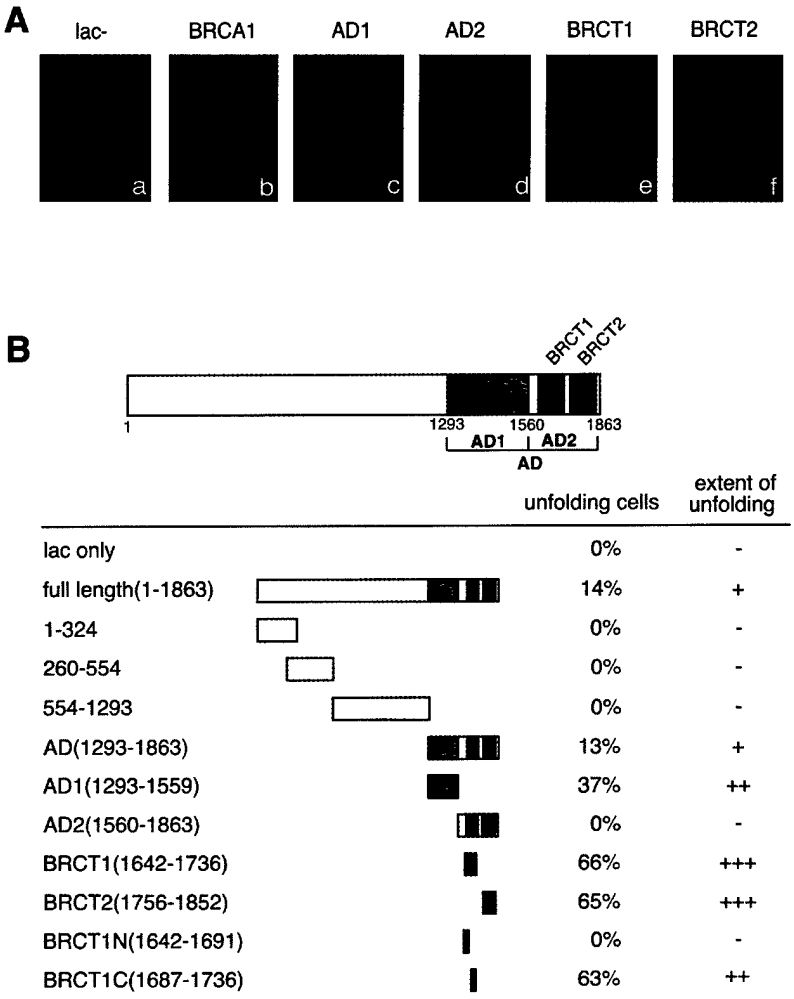


Figure 1. **BRCA1 induces large-scale chromatin decondensation.** (A) The AO3_1 CHO cell line was transiently transfected with expression vectors for the following proteins: lac repressor (a), lac–BRCA1(b), lac–AD1(c), lac– AD2(d), lac–BRCT1(e), and lac–BRCT2(f). A polyclonal anti–lac repressor antibody and a Cy3-conjugated secondary anti–rabbit IgG were used for immunostaining. Nuclei were visualized by DNA staining with DAPI. (B) The ability of various BRCA1 fragments to unfold chromatin was measured by the percentage of transfected cells that displayed enlarged lac staining and the degree of unfolding. Over 100 transfected cells were surveyed for each construct. Single, double, and triple plus signs indicate various degrees of chromatin unfolding, as exemplified by images for lac–BRCA1 (+), lac–AD1 (++), and lac–BRCT1 (+++). Also shown are schematic diagrams and amino acid coordinates for various BRCA1 fragments.

VP16 AAD, is not yet understood. It may represent a combination of several factors, including cell cycle–dependent expression as well as the nature of the qualitative assay employed. Transgene arrays have been shown to display coordinated gene silencing effects that are accompanied by cooperative changes in chromatin structure across the entire array (Pikaart et al., 1998). These changes in gene expression and chromatin structure show a variegating phenotype that is clonally inherited. Therefore, it is possible that the large-scale chromatin decondensation induced by a transcriptional activator in the lac system may also display cooperative and variegating responses.

Deletion analysis showed that chromatin-unfolding activity was conferred by the last 570 aa of BRCA1 (Fig. 1 B, aa 1293–1863). This region of BRCA1, previously designated AD (Hu et al., 1999), consists of two subdomains that act synergistically to stimulate transcription (Fig. 1 B, AD1, aa 1293–1559, and AD2, aa 1560–1863). As illustrated in Fig. 1 B, AD2 contains the two BRCT repeats, BRCT1 and BRCT2. Further domain mapping indicated that AD1, BRCT1, and BRCT2 could independently induce large-scale chromatin unfolding (Fig. 1 A, c, e, and f, and B). It is of note that AD1 often leads to a ball-shaped structure with smooth edges, whereas BRCT1 and BRCT2 tend to give rise to more extended, fiber-like structures with irregular shapes (Fig. 1 A, compare c with e and f). The degree of unfolding by BRCT1 and BRCT2 approached that observed with VP16, with >60% of cells showing this response, whereas the AD1 subdomain showed intermediate unfolding. Interestingly, both the magnitude of this unfolding and the percentage of cells showing unfolding using these subdomains was significantly higher than observed using the full-length BRCA1 fusion protein (Fig. 1 B). Furthermore, AD2, which includes both the BRCT1 and BRCT2 repeats, failed to cause obvious decondensation of high-order chromatin structure (Fig. 1 A, compare d with e and f). As explained below, we interpret this as an indication of a negatively regulated chromatin unfolding activity associated with the full-length BRCA1 and AD2 region.

Further dissection of the BRCT1 domain shows that the 50-aa COOH-terminal half of BRCT1 is sufficient for inducing maximal chromatin unfolding (Fig. 1 B, BRCT1C). In contrast, the NH_2-terminal half of BRCT1 (BRCT1N) with a comparable size to BRCT1C, fails to mediate any chromatin decondensation. Furthermore, none of the BRCA1 fragments upstream of AD displayed any activity in chromatin unfolding (Fig. 1 B, 1–324, 260–554, and 554–1293), although they were expressed at similar levels as the chromatin-unfolding domains (unpublished data and see Fig. 5 B). Previous studies have shown that these regions upstream of AD are responsible for BRCA1 interactions with various proteins or protein complexes. For example, the NH_2-terminal region

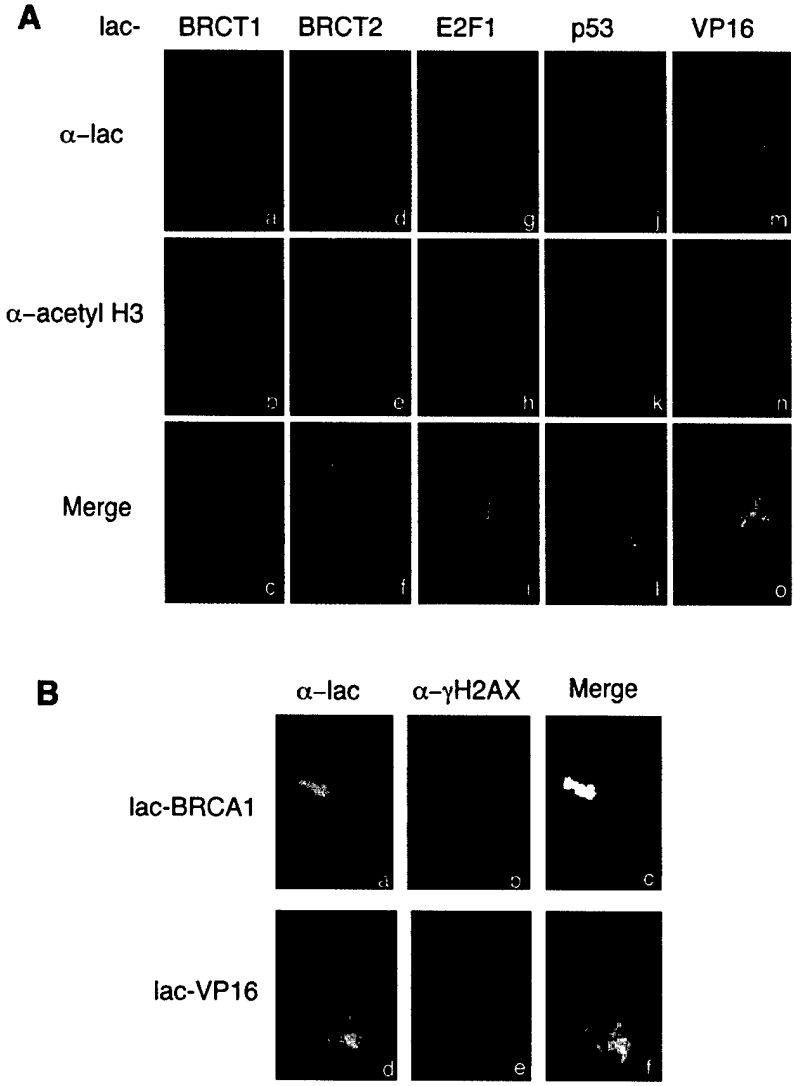


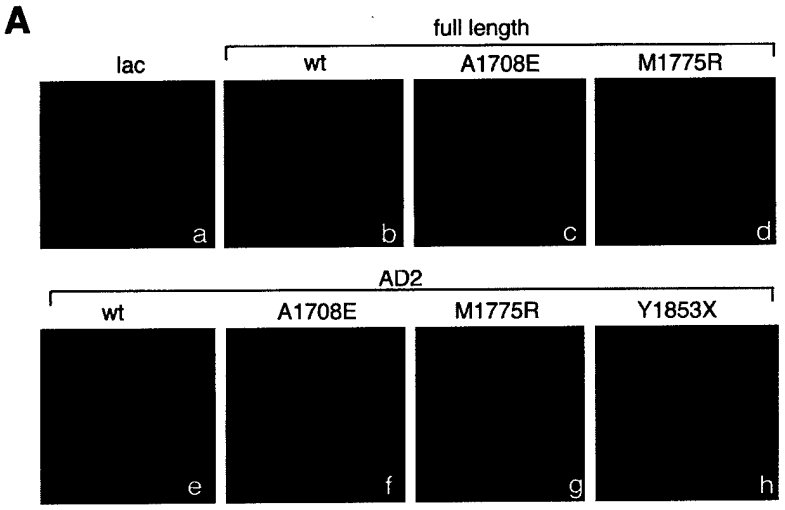
Figure 2. **Comparison of chromatin unfolding by various lac fusion proteins.** (A) Absence of histone hyperacetylation associated with BRCT-mediated chromatin unfolding. AO3_1 cells were transfected with the expression vectors for lac fused with BRCT1 (a–c), BRCT2 (d–f), E2F1 (g–i), p53 (j–l), or VP16 (m–o). The lac (green), acetylated histone H3 (red), and the merged images were captured by confocal immunofluorescence microscopy. (B) Association of lac–BRCA1 with phosphorylated H2AX. AO3_1 cells were transfected with the lac–BRCA1 expression vector. Cells were double stained with the mouse anti-lac antibody and a rabbit anti–γ-H2AX antibody (1:100 dilution; Upstate Biotechnology).

of BRCA1 binds BARD1 (Wu et al., 1996), whereas the central region of the protein mediates BRCA1 interactions with hSNF/SWI (Bochar et al., 2000), RAD50/MRE11/NBS1 (Zhong et al., 1999), and RAD51 (Scully et al., 1997b). The inability of these regions to induce large-scale chromatin decondensation argues that chromatin unfolding is not simply due to recruitment of any large proteins or protein complexes to the lac binding sites. Rather, the chromatin unfolding activity is conferred by three specific subdomains in the transactivation domain of BRCA1, suggesting that chromatin decondensation is related to BRCA1 functions in transcriptional regulation and DNA repair.

Distinction between BRCT and other well-characterized transactivation domains in large-scale chromatin unfolding

A previous study has shown that VP16-induced chromatin unfolding is accompanied by recruitment of histone acetyltransferases and local histone hyperacetylation, a property frequently observed for transcriptionally active or competent chromatin (Tumbar et al., 1999) (Fig. 2 A, m–o). Here we extended the previous work by examining the transactivation domains of two cellular transcription factors, E2F1 and p53. Like lac-VP16, lac-E2F1 and lac-p53 also induced significant chromatin unfolding in 60 and 45% of transfected cells, respectively (Fig. 2 A, g and j). Furthermore, the lac-E2F1– and lac-p53–unfolded chromatin regions were enriched with hyperacetylated histone H3 and H4 (Fig. 2 A, g–i and j–l, and unpublished data). Thus, all three well-characterized transactivation domains (VP16, E2F1, and p53) can simultaneously induce large-scale chromatin unfolding and histone hyperacetylation. However, it remains unknown whether the observed histone hyperacetylation is causally related to chromatin unfolding.

The extent of chromatin decondensation induced by a single BRCT repeat is comparable to that exhibited by these potent transcriptional ADs (Fig. 2 A, compare a and d with g, j, and m). However, no obvious histone H3 or H4 hyperacetylation was detected in the BRCT1- or BRCT2-unfolded chromatin regions (Fig. 2 A, a–c and d–f), suggesting that BRCT-mediated chromatin unfolding is a separable event from histone acetylation. Although both BRCT repeats are required for AD2-mediated transcriptional activation, a single repeat does not serve as a strong AD (Chapman and Verma, 1996; Monteiro et al., 1996) (unpublished data).



B

		unfolding cells	extent of unfolding
	lac only	0%	-
	BRCA1wt	14%	+
(a)	Q563X	0%	-
(a)	E908X	0%	-
(b)	C61G	19%	+
(b)	S1040N	9%	+
(b)	R1347G	12%	+
(b)	R1443X	14%	+
(c)	A1708E	59%	++
(c)	1756insC	38%	+
(c)	M1775R	79%	++
(c)	R1835X	51%	++
(c)	Y1853X	47%	++

Figure 3. **A subset of cancer-predisposing mutations in the COOH-terminal domain of BRCA1 cause increased chromatin unfolding.** (A) Cancer-predisposing mutations were introduced into either the full-length BRCA1 (a–d) or AD2 (e–h). The corresponding expression vectors were transfected into AO3_1 cells, and immunostaining was performed as described in Fig. 1. (B) Summary of the effects of different cancer-associated mutations on chromatin unfolding. All mutants shown in this table were tested in the context of full-length BRCA1. Locations of missense mutations are indicated by asterisks, whereas those of nonsense and frameshift mutations are indicated by wavy lines. All mutations are grouped into three (a–c) as discussed in the text.

Therefore, chromatin unfolding by BRCT may be a necessary, but not sufficient step, in transcriptional activation.

In addition to acetylation, histones are subject to other posttranslational modifications under various physiological conditions. Of particular interest, phosphorylation of H2AX, a histone H2A variant, at serine 139 (γ-H2AX) is rapidly stimulated following ionizing radiation (Rogakou et al., 1999). Before irradiation, a subset of γ-H2AX nuclear foci colocalize with BRCA1 foci (Paull et al., 2000). After DNA damage, the number of both γ-H2AX and BRCA1 nuclear foci increases significantly; furthermore, the majority of BRCA1 foci overlapped γ-H2AX foci (Paull et al., 2000). These observations most likely reflect localized recruitment of the putative H2AX kinase and phosphorylation of H2AX-containing nucleosomes that are already present at these sites, rather than recruitment of new H2AX protein.

Using an antibody that specifically recognizes the phosphorylated form of H2AX (γH2AX), we detected colocalization of the endogenous γ-H2AX with the full-length BRCA1 fusion protein in a sub-population (15%) of the lac–BRCA1-transfected cells (Fig. 2 B, a–c). In contrast, lac-VP16 did not display any colocalization with γ-H2AX (Fig. 2 B, d–f), nor did lac-BRCT1 or lac-BRCT2 (unpublished data). It is not clear whether phosphorylation of H2AX is causally linked to BRCA1-mediated chromatin unfolding, as γ-H2AX colocalization is also observed in lac–BRCA1-expressing cells that do not display chromatin decondensation. Consistent with previous reports (Rogakou et al., 1999; Paull et al., 2000), ionizing radiation significantly increased the number and overall intensity of γ-H2AX foci (unpublished data). However, the strong γ-H2AX signal over the entire nucleus made it difficult to examine the effect of DNA damage on the colocalization between γ-H2AX and lac-BRCA1 at the lac binding sites.

Work by Paull et al. (2000) has shown that H2AX at the damaged sites is rapidly phosphorylated after ionizing radiation, which is followed later by colocalization of BRCA1 and other repair proteins (Paull et al., 2000). It is possible that the putative kinase(s) responsible for H2AX phosphorylation directly binds to the full-length BRCA1. In such an

event, tethering lac–BRCA1 may simply bring the kinase(s) to the tandem array of the lac binding sites, thus causing hyperphosphorylation of H2AX present in the surrounding chromosomal region. Whereas the functional significance of H2AX phosphorylation in chromatin unfolding remains to be explored, our finding is consistent with the previous suggestion of a physical link between γ-H2AX and BRCA1.

Allele-specific effects of cancer-predisposing mutations of BRCA1 on chromatin unfolding

To determine the effect of cancer-associated mutations on the BRCA1-dependent chromatin unfolding, we introduced a series of common cancer-predisposing mutations into either full-length BRCA1 or AD2. Based on their behaviors in the chromatin-unfolding assay, mutations were classified into three phenotypic categories. The first includes nonsense mutations resulting in truncation of the entire COOH terminus (Fig. 3 B, a). According to previous studies, BRCA1 mutants that lack the COOH terminus of the protein are defective in stimulating transcription and DNA repair (Somasundaram et al., 1997; Abbott et al., 1999; Scully et al., 1999; Jin et al., 2000). As shown in group a of Fig. 3 B, these COOH-terminal truncation mutants also failed to induce chromatin unfolding. The second group of mutants include missense mutations that are located upstream of AD2 (group b, i.e., C61G, S1040N, and R1347G). None of the mutants in this group significantly affects BRCA1-mediated chromatin unfolding.

Contrary to the behaviors of first two groups, mutations in group c markedly enhanced the ability of lac-BRCA1 to induce chromatin unfolding (Fig. 3 B). For example, A1708E, M1775R, and Y1853X led to a pronounced enlargement of the unfolded chromatin structure (Fig. 3 A, compare b with c and d, and e with f–h). The same mutations also significantly increased the percentage of transfected cells that showed chromatin unfolding (Fig. 3 B). For instance, 79% of the cells that expressed the M1775R mutant displayed significant chromatin unfolding, compared with 14% for the wild-type full-length protein. This is an even higher percentage than that previously observed for the VP16 activator (Tumbar et al., 1999).

Interestingly, all mutations in group c result in single aa substitutions or small deletions within the AD2 region. Many of the mutations in this group have been shown previously to abolish AD2 interactions with other transcription-related proteins, including the RNA pol II holoenzyme (Scully et al., 1997a; Neish et al., 1998; Yarden and Brody, 1999). As discussed below, by retaining the chromatin unfolding activity of BRCA1 but blocking its role in other steps of transcriptional activation, these mutations in group c may lead to accumulation of the highly decondensed chromatin structure as observed in the unfolding assay.

Identification of a novel BRCA1-interacting protein

Application of the chromatin unfolding assay allowed us to identify a large-scale chromatin unfolding activity associated with BRCA1, and to narrow down the chromatin-unfolding region of BRCA1 to small subdomains in the COOH terminus of the protein. To identify cofactors recruited by the BRCT repeats to mediate chromatin unfolding, we used BRCT1 as the bait in a yeast two-hybrid screen. One candidate gene, cofactor *COBRA1*, was isolated from a human ovary cDNA library. It encodes a novel 580-aa protein rich in leucine residues (17%) (Fig. 4). COBRA1 also contains three repeats of the LXXLL motif, often present in many transcription coactivators and responsible for mediating their ligand-dependent interactions with steroid hormone receptors (Heery et al., 1997). Database searches revealed COBRA1-related hypothetical proteins in mice and flies that share 96 and 51% aa identity with the human protein, respectively (Fig. 4).

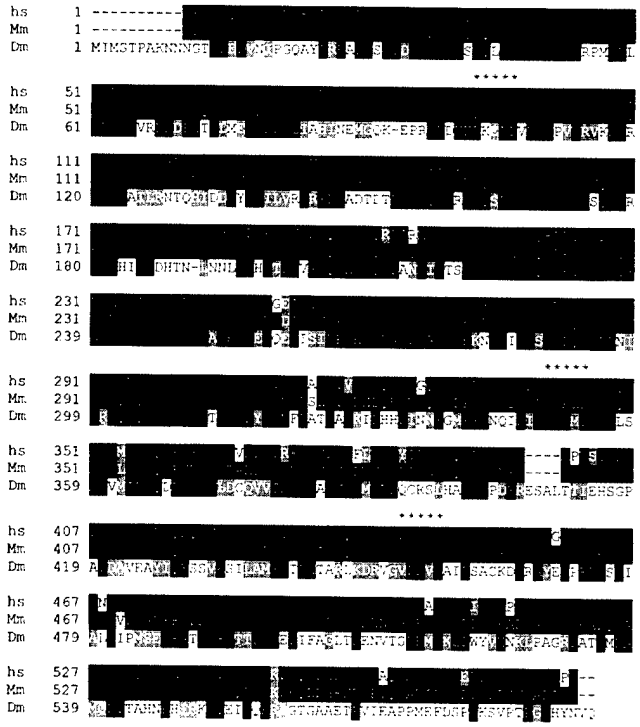


Figure 4. **Sequence alignment of human COBRA1 and its homologues from mice and flies.** The conserved aa residues are highlighted in black, and the similar residues in gray. The locations of the LXXLL motif are indicated by asterisks.

To confirm the interaction between BRCA1 and COBRA1, a lysate of human HEK293T cells that ectopically expressed FLAG-tagged COBRA1 was immunoprecipitated with an anti-FLAG antibody, followed by immunoblotting with an anti-BRCA1 antibody. As shown in Fig. 5 A, the endogenous human BRCA1 was coprecipitated in a FLAG-COBRA1–dependent manner (lanes 1 and 2). As a control, addition of an excess of FLAG peptide to the immunoprecipitation reaction abolished the BRCA1 signal in the immunoprecipitate (Fig. 5 A, lane 3).

To further assess the binding specificity of COBRA1 to the BRCT1 region of BRCA1, we cotransfected HEK293T cells with FLAG-COBRA1 and lac repressor fused with various fragments of BRCA1. The cell lysates were then immunoprecipitated with the anti-FLAG antibody and subsequently immunoblotted with the anti-lac antibody. As shown in Fig. 5 B, lac-BRCT1 was capable of binding to the FLAG-COBRA1 (lane 2). Consistent with their activity in

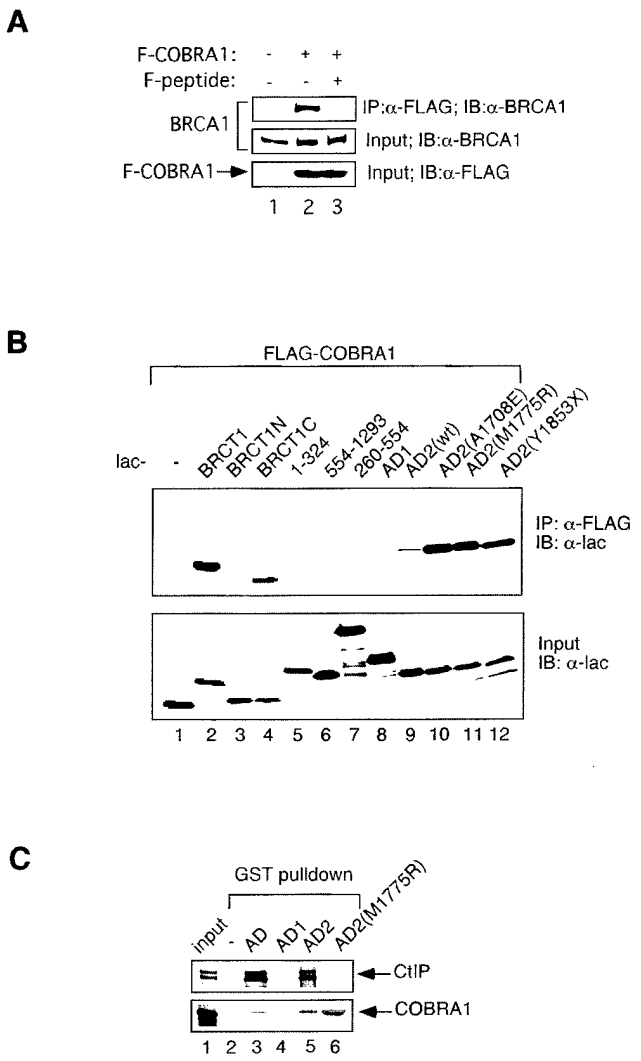


Figure 5. **Identification of COBRA1 as a novel BRCA1-interacting protein.** (A). COBRA1 interacts with endogenous full-length BRCA1. Human HEK293T cells were transfected with either an empty vector (lane 1) or expression vector for the FLAG-tagged COBRA1 (F-COBRA1; lanes 2 and 3). Cell lysates were immunoprecipitated (IP) with an anti-FLAG antibody conjugated to Protein A agarose beads (Sigma-Aldrich), in the absence (lane2) or presence (lane 3) of the FLAG peptide at a final concentration of 0.8 μg/ml. The immunoprecipitates were immunoblotted (IB) with a monoclonal anti-BRCA1 antibody (AB1 from Oncogene), the results of which are shown in the top panel. As controls, the crude lysates were immunoblotted for the endogenous BRCA1 (middle) and the ectopically expressed FLAG-COBRA1 (bottom). (B) Further characterization of the interaction between BRCA1 and COBRA1. Various lac–BRCA1 fusion constructs and the FLAG-COBRA1 expression vector were cotransfected into HEK293T cells. Cell lysates were immunoprecipitated with an anti-FLAG antibody and subsequently immunoblotted with an anti-lac antibody, the results of which are shown in the top panel. Expression of the lac fusion proteins was determined by immunoblotting of the crude lysates with the anti-lac antibody (bottom). (C) In vitro GST pull-down assay to characterize the BRCA1-COBRA1 interaction. Various GST fusion proteins were expressed in bacteria and coupled to glutathione agarose beads (unpublished data). An equal amount of the GST fusion proteins was used to pull down the ^{35}S labeled, in vitro translated CtIP (top) or COBRA1 (bottom).

chromatin unfolding, the COOH-, but not the NH_2-terminal half of BRCT1 (Fig. 1 B), interacted with COBRA1 (lanes 3 and 4). None of the BRCA1 fragments upstream of the BRCT repeat, including AD1, displayed any significant affinity for COBRA1 (lanes 5–8). Taken together, our data show that the COBRA1 binding correlates with the BRCT1-mediated large-scale chromatin unfolding.

As shown in Fig. 3, cancer-predisposing mutations in the 3′ region of *BRCA1* caused significant enhancement of the chromatin unfolding activity. Intriguingly, the same mutations (A1708E, M1775R, and Y1853X) also increased the affinity for COBRA1 in the coimmunoprecipitation assay (Fig. 5 B, compare lane 9 with lanes 10–12). A similar result was also observed in an in vitro glutathione *S*-transferase (GST) pull-down assay (Fig. 5 C). In this case, ^{35}S-labeled, in vitro–translated COBRA1 was pulled down by both GST–AD and GST–AD2, but not by GST–AD1 (Fig. 5 C, bottom panel, lanes 3–5). Furthermore, COBRA1 displayed a higher affinity for the mutant (M1775R) than the wild-type GST–AD2 fusion (Fig. 5 C, bottom panel, lanes 5 and 6). As a control, we also used ^{35}S-labeled CtIP, a transcriptional corepressor that binds to the COOH terminus of BRCA1 (Wong et al., 1998; Yu et al., 1998; Li et al., 1999). Consistent with previous findings, CtIP binds specifically to AD2 but, unlike COBRA1, its association with AD2 is abolished by the M1775R mutation (Fig. 5 C, top panel, lanes 5 and 6). Thus, the same cancer-predisposing mutations exert opposite effects on BRCA1 binding to two different partners.

Involvement of COBRA1 in BRCT1-mediated chromatin unfolding

To explore the role of COBRA1 in the BRCT1-mediated chromatin unfolding, we cotransfected FLAG-COBRA1 with various lac–BRCA1 fusion constructs into AO3_1 cells. As detected by confocal immunofluorescent microscopy, FLAG-COBRA1 and lac–BRCT1 colocalized in 96% of the cells that expressed both proteins (Fig. 6 A, a–c). In contrast, we did not detect any enrichment of the FLAG-COBRA1 signal at either the BRCT2- or AD1-unfolded chromatin regions (Fig. 6 A, BRCT2, d–f, and AD1, g–i). Thus, whereas all three subdomains are capable of inducing large-scale chromatin unfolding, they appear to recruit distinct cofactors to mediate this process.

Wild-type AD2, which failed to induce chromatin unfolding (Fig. 1), did not display any obvious colocalization with COBRA1 (Fig. 6 A, panels j–l). However, two 3′ cancer-predisposing mutations in the same context led to pronounced recruitment of COBRA1 to the unfolded chromatin regions (Fig. 6 A, A1708E, m–o, and M1775R, p–r). Colocalization of COBRA1 and the mutant lac–AD2 fusion proteins was observed in >90% cells that expressed both proteins. Thus, the effect of the 3′ mutations on COBRA1 recruitment correlates with their stimulatory effects on chromatin unfolding.

To directly assess the impact of COBRA1 on large-scale chromatin structure, we used lac repressor to target COBRA1 to the lac binding sites in AO3_1 cells. As shown in Fig. 6 B, 61% of the cells that expressed lac–COBRA1 showed a comparable extent of chromatin unfolding as did lac–BRCT1

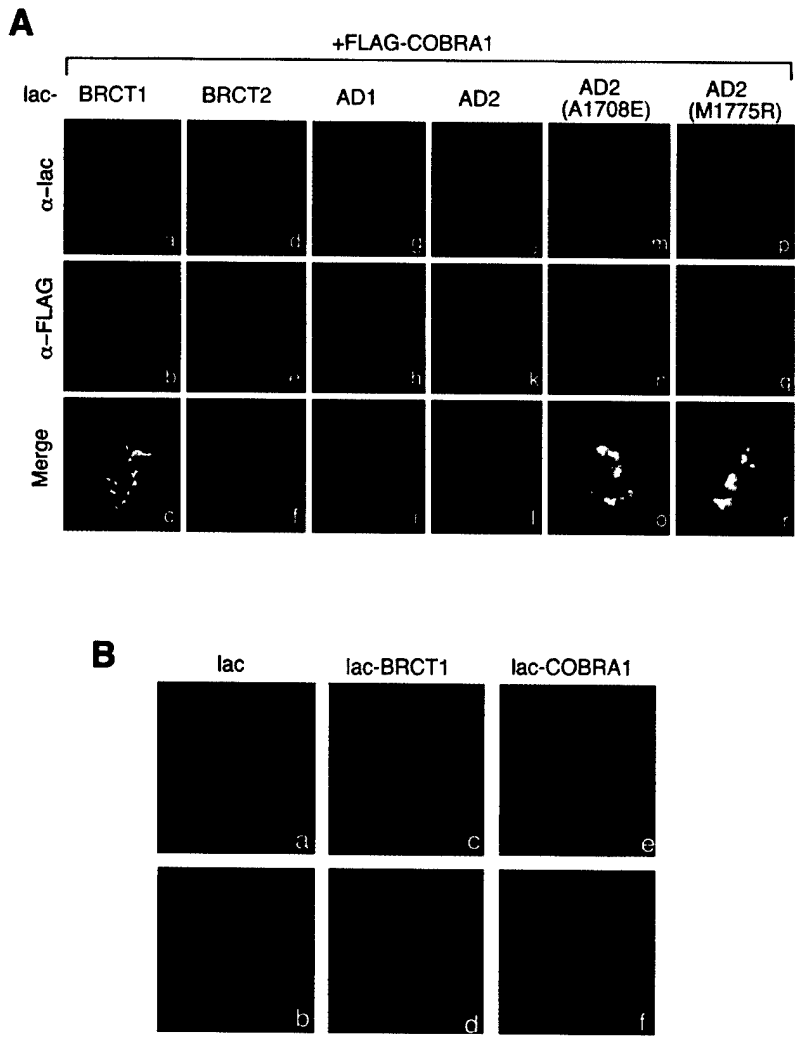


Figure 6. **COBRA1 colocalizes with lac–BRCT1 and can induce large-scale chromatin unfolding.** (A) Colocalization of lac fusion proteins (red) and FLAG-COBRA1 (green) at the unfolded chromatin region. AO3_1 cells were cotransfected with the expression vectors for FLAG-COBRA1 and lac fused with various fragments of BRCA1. The images were captured by confocal immunofluorescence microscopy. (B) COBRA1 induces chromatin unfolding when directly targeted to the chromosome. AO3_1 cells were transfected with the expression vectors for lac repressor alone (a and b), lac–BRCT1 (c and d), or lac–COBRA1 (e and f). Chromatin unfolding was detected as described in the previous figures.

(compare c and d with e and f). This finding strongly implicates COBRA1 in BRCT1-mediated chromatin restructuring.

Discussion

Eukaryotic genomes are packaged through multiple steps into higher levels of chromatin structure. It is now well established that remodeling of local chromatin structure is a key step common to the initiation of multiple chromosomal functional events, including transcription, DNA replication, repair, and recombination (Elgin and Workman, 2000; Fyodorov and Kadonaga, 2001). Whereas intense research in the past decade has provided a wealth of information regarding the biochemical basis for chromatin remodeling at the nucleosome level, much less is known about reorganization of higher levels of chromatin structure. It remains unclear whether the known modifications of nucleosome organization are sufficient for changes in large-scale chromatin organization, or whether novel mechanisms acting at higher levels of chromatin structure are responsible for changes in large-scale chromatin organization.

A major difficulty in distinguishing these two possibilities is that most assays for identifying transcriptional activators or coactivators have used transcriptional activity as a final readout. Direct assays for changes in higher order chromatin structure have not been used previously. Because BRCA1 had been functionally implicated in a range of nuclear processes, it was reasonable to postulate that the regulation of these multiple nuclear events might occur through a general chromatin remodeling activity of BRCA1. The lac repressor–tethering system, while artificial in many aspects, provided an excellent assay to pursue this research direction.

Our findings in this current study strongly suggest that BRCA1 recruits COBRA1, a novel factor, to the lac operator–containing chromatin region. Within the constraints of the lac repressor–tethering assay, BRCA1-dependent unfolding of higher levels of chromatin structure appears to be at least partially mediated through recruitment of COBRA1. Notably, BRCA1-mediated chromatin decondensation is distinct from transcriptional activation per se and histone hyperacetylation. It is unclear how unique the histone acetylation–independent chromatin unfolding is. Although the chromatin unfolding produced by VP16, E2F1, and p53 is accompanied by histone hyperacetylation, no causal relationship between histone acetyltransferases recruitment and chromatin unfolding has been demonstrated. Moreover, preliminary data suggests that large-scale decondensation produced by estrogen receptor does not correlate with histone hyperacetylation (A. Nye and A. Belmont, personal communication).

Whereas the lac-based chromatin-unfolding assay provides a new tool for visualizing chromatin dynamics and in vivo protein–protein interactions in mammalian cells, it is important to point out that the molecular and biochemical basis for BRCA1-mediated chromatin decondensation is yet to be understood. Furthermore, utilization of a long tandem array of lac binding sites may raise the concern that the observed chromatin unfolding could simply be due to steric effects of the proteins/protein complexes that are brought to the lac binding sites. However, we believe this possibility is unlikely because our work does not indicate an obvious correlation between the potency of chromatin unfolding and the size or charge of the tethered protein fragments. For example, the minimal chromatin-unfolding domain defined in our study is only 50 aa long (BRCA1C). In contrast, several other BRCA1 fragments that range in size from 324 to 740 aa do not display any chromatin-unfolding activity (Fig. 1 B). In addition, BRCT1 and BRCT2 have a net charge of +5 and –6, respectively, yet both demonstrate strong chromatin-unfolding activity. On the other hand, BRCT1N carries more positive charges (+5) than BRCT1C (+1), but only the latter can induce chromatin decondensation. Finally, in previous work using either lac repressor tetramer, or lac repressor fused to several other protein domains up to ~350 aa in size (i.e., GFP), no effect on large-scale chromatin structure has been observed (Robinett et al., 1996, and A. Belmont, personal communication).

In our minds, a more serious caveat concerning the lac repressor–tethering system is the question of whether the observed effects produced by BRCA1 and other proteins on large-scale chromatin unfolding are physiologically relevant given the high numbers of lac operator repeats involved. In fact, the exact number of lac repressors binding per lac operator has not been determined and there is reason to believe that lac repressor binding may be significantly limited by steric constraints and phasing of lac operators relative to the nucleosome linker DNA. However, we note that a recent study on a transgene array containing a viral promoter with several glucocorticoid hormone response elements observed a very similar type of large-scale chromatin decondensation produced by glucocorticoid receptor (Muller et al., 2001). Ultimately, validation of the physiological significance of our observations of BRCA1-dependent large-scale chromatin unfolding will depend on the outcome of future experiments exploring the mechanisms of unfolding and identifying the biological functions of other transacting factors involved, such as COBRA1.

With these caveats in mind, we find it particularly intriguing that a subset of cancer-predisposing mutations of BRCA1 lead to increased chromatin unfolding and recruitment of COBRA1. Although the genotype–phenotype relationship in cancer-predisposing mutations of BRCA1 remains to be understood, it is generally assumed that most, if not all, BRCA1 mutations lead to loss of the biological functions of the protein. However, the behaviors of the BRCA1 mutants in the chromatin-unfolding assay clearly demonstrate an allele-specific effect. Consistent with this finding, it has been reported that mutations at different locations along the coding sequence of BRCA1 differentially affect the penetrance of BRCA1-dependent breast and ovarian cancer (Gayther et al., 1995; Risch et al., 2001). It remains to be determined whether the three groups of mutations that cause differential effects on chromatin unfolding (Fig. 3) may indeed lead to distinct clinical consequences in terms of risks, types, or prognosis of BRCA1-associated cancers. In particular, it will be interesting to see whether those 3′ mutations that enhance chromatin unfolding exhibit any dominant or semidominant phenotype in cancer genetics. It is conceivable that constitutive decondensation of large-scale chromatin structure may cause additional deleterious effects on genome stability and thus result in more severe clinical consequences in cancer development.

Our study also indicates that BRCT-mediated chromatin unfolding may be tightly regulated. As shown in Figs. 1 and 3, a single BRCT motif is more potent in chromatin unfolding than the larger fragments of the protein that contain both BRCT repeats. Furthermore, the full-length wild-type BRCA1 only exhibits a moderate chromatin-unfolding activity, whereas the cancer-predisposing mutations in group c (Fig. 3 B) that affect the integrity of the BRCT repeats significantly enhance the chromatin-unfolding activity and COBRA1 binding. These results lead us to the following two models that could explain negative regulation of BRCA1-mediated chromatin unfolding. In a "trans-inhibition" model, we speculate that binding of a putative inhibitor (i.e., CtIP) to AD2 region of BRCA1 may prevent BRCA1 from interacting with its cofactors for chromatin unfolding (i.e., COBRA1). In an alternative, "cis-inhibition" model, the two BRCT tandem repeats may form an intramolecular dimer. This in turn may reduce the affinity of both BRCT repeats for their corresponding cofactors. Conceivably, the "superactivating" mutations in group c may prevent binding of the putative inhibitor or the intramolecular interaction between the two BRCT motifs, thus rendering the protein constitutively active for binding to the cofactors that mediate chromatin unfolding.

It is plausible that BRCT-mediated chromatin unfolding may lead to a novel nuclear function of BRCA1 in global reorganization of the genome. However, in light of the known function of the COOH-terminal region of BRCA1 in transcription and DNA repair, the observed chromatin decondensation may represent the first step in BRCA1-mediated regulation of these two nuclear processes (Fig. 7). In such a

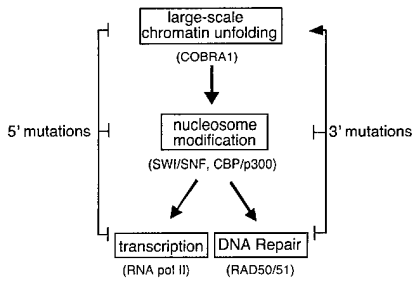


Figure 7. **Model for BRCA1-mediated nuclear functions.** Inhibitory and stimulatory effects of the 5′ and 3′ mutations on the three steps are indicated by bars and arrows on the sides, respectively. Factors in parentheses are those that may be targeted or recruited by BRCA1 to facilitate a specific step in activation of transcription or DNA repair. See text for more detail.

model, higher order chromatin decondensation may be followed by BRCA1-mediated chromatin modification at the nucleosomal level (i.e., histone hyperacetylation) and recruitment of the transcription or repair machineries. As shown in Fig. 7, nonsense mutations that result in truncation of the entire COOH-terminal region (5′ mutations) may abolish BRCA1 functions in all three steps, resulting in a completely inactive mutant protein. On the other hand, mutations located at the 3′ end of the gene (3′ mutations) may render BRCA1 incompetent at the second and third steps, but still allow constitutive chromatin decondensation at the first step. This could then lead to accumulation of extensively unfolded chromatin structure as seen in our study. Consistent with this model, many 3′ cancer-predisposing mutations abolish BRCA1 interactions with RNA pol II holoenzyme and the histone modifying enzymes (Scully et al., 1997a; Neish et al., 1998; Yarden and Brody, 1999), as well as nucleosome remodeling in yeast (Hu et al., 1999). Thus, chromatin unfolding may be a necessary but not sufficient step for BRCA1-dependent transcriptional activation. Additional steps such as histone modification and recruitment of the basal machinery may also be required for fulfilling BRCA1 function in transcription and DNA repair.

Materials and methods

Chromatin unfolding assay

To construct the EGFP-lac-E2F1 and EGFP-lac-p53 fusion expression vectors, the PCR fragments that encode the E2F1 (aa 368–437) and p53 (aa 1–73), respectively, were cloned into the AscI site in the plasmid p3′SS d tb CI EGFP AscI (NYE4) (A.C. Nye and A.S. Belmont, personal communication). The correct orientation of the inserts was identified by colony hybridization and confirmed by DNA sequencing. To construct the lac-BRCA1 plasmids, the sequence for lac repressor was first amplified by PCR from the plasmid NYE4. The lac sequence was cloned into the HindIII–NotI sites of pRC-CMV (Invitrogen), generating pRC-lac. Various BRCA1 fragments and the COBRA1 sequence were amplified by PCR and inserted into the unique AscI site of pRC-lac.

The chromatin unfolding experiments were performed as previously described (Tumbar et al., 1999). Briefly, AO3_1 cells were transiently transfected with the lac expression vectors using the FuGENE 6 transfection reagent (Roche). The medium was changed 24 h after transfection and cells were immunostained 48 h after transfection. Cells grown on glass coverslips were fixed with 1.6% paraformaldehyde for 30 min in PBS, permeabilized with 0.2% Triton X-100 in PBS for 5 min, and blocked in 1% normal goat serum in PBS for 1 h. The coverslips were then incubated with primary antibodies at room temperature for 1 h, followed by incubation with the appropriate secondary antibodies for 1 h. Unless otherwise specified, a rabbit polyclonal anti–lac repressor antibody (Stratagene) and mouse monoclonal anti-FLAG antibody (Sigma-Aldrich) were applied at 1:20,000 dilution. The anti–acetylated histone H3 antibody was raised against di-acetylated H3 (Lys9 and Lys14) (Boggs et al., 1996) (Lin et al., 1989), a gift from Drs. C. Mizzen and C.D. Allis (University of Virginia, Charlottesville, VA). The secondary antibodies were goat anti–rabbit IgG-conjugated with Cy3 (Amersham), and horse anti–mouse IgG-conjugated with fluorescein isothiocyanate (FITC; Vector Laboratories).

For visualization of the nuclei, cells were stained with 0.2 μg/ml 4,6-diamidino-2-phenylindole (DAPI) for 5 min before mounting. Fluorescent images were acquired by a charged-coupled device camera (Hamamatsu ORCA) that was mounted on a Nikon Microphot-SA microscope and equipped with Improvision Openlab software. Confocal images were collected on a Zeiss LSM410 confocal microscope. Figs. were assembled using Adobe Photoshop (v. 5.5).

Yeast two-hybrid screen

To identify proteins that specifically interact with the BRCT1 repeat of BRCA1, the standard yeast two-hybrid screen was performed in the following manner. First, the bait plasmid was generated by inserting a PCR-amplified cDNA fragment encoding the BRCT1 sequence (aa 1642–1736) into the NdeI–EcoRI restriction sites of pAS2–1 (CLONTECH Laboratories, Inc.), resulting in an in-frame fusion with the GAL4 DNA-binding domain. The resultant plasmid, pAS2-BRCT1, and a human ovary cDNA prey library (CLONTECH Laboratories, Inc.) were sequentially transformed into the *S. cerevisiae* strain CG1945 according to the manufacturer's instructions (CLONTECH Laboratories, Inc.). Transformants were plated on synthetic medium lacking tryptophan, leucine and histidine but containing 1 mM 3-aminotriazole. Approximately 2.3 million transformants were screened. The candidate clones were retrieved from the yeast cells and reintroduced back to the same yeast strain to verify the interaction between the candidates and the BRCT1 bait. The specificity of the interaction was determined by comparing the interactions between the candidates and various bait constructs.

Coimmunoprecipitation

HEK293T cells were transfected using LipofectAmine 2000 (GIBCO BRL). 24 h after transfection, cells were washed twice with PBS and lysed in 0.5 ml lysis buffer (50 mM Hepes, pH 8, 250 mM NaCl, 0.1% NP-40, and protease inhibitor tablets from Roche). After brief sonication, the lysate was centrifuged at 16,000 *g* for 12 min at 4°C. The supernatant was used for subsequent coimmunoprecipitation. 20 μl of the supernatant was used as crude extract for detecting protein expression level. 15 μl of a 50% slurry of the anti-FLAG agarose beads (Sigma-Aldrich) was used in each immunoprecipitation. Immunoprecipitation was performed overnight at 4°C. The beads were centrifuged at 3,300 rpm for 2 min, and washed three times with washing buffer (50 mM Hepes, pH8, 500 mM NaCl, 0.5% NP-40) and three times with RIPA buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1% NP-40, 0.1% SDS, and 0.5% sodium deoxycholate). Each wash was performed for at least 30 min. The precipitates were then eluted in 15 μl 2× SDS-PAGE sample buffer. Gel electrophoresis was followed by immunoblotting according to standard procedures.

GST pulldown assay

The PCR fragments encoding various BRCA1 fragments were cloned into pGEX-2T and the constructs were confirmed by sequencing. The GST-BRCA1 proteins were made and purified, with the induction of protein expression performed at 19°C overnight. pcDNA3 vector containing the COBRA1 gene was used for in vitro transcription and translation in the TnT Reticulocyte Lysate system (Promega). The ^{35}S-labeled COBRA1 was translated in vitro according to the manufacturer's instructions and mixed with 10 μg the GST-bound bead in 0.5 ml binding buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.3 mM DTT, 0.1% NP-40 and protease inhibitor tablet). The binding reaction was performed at 4°C overnight and the beads were subsequently washed four times with washing buffer (same as binding buffer except 0.5% NP-40 was used), 30 min each time. The beads were eluted in 10 μl 2 × SDS-PAGE sample buffer and the proteins were resolved on 10% denaturing gel. The gel was then dried and exposed to x-ray films for overnight.

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JunB potentiates function of BRCA1 activation domain 1 (AD1) through a coiled-coil-mediated interaction

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BRCA1 is involved in the regulation of multiple nuclear events including transcription. AD1, one of the two *trans*-activation domains in BRCA1, stimulates transcription in a cell context-dependent manner. Here, it is shown that BRCA1 interacts with Jun proteins via a coiled-coil motif in AD1 and the basic leucine zipper (bZIP) region of the Jun proteins. The Jun-interacting domain in BRCA1 is critical for AD1-mediated transcriptional activation. In particular, the strength of AD1 in transcriptional activation is limited by the JunB level and ectopic expression of JunB potentiates the transcriptional activity of AD1. Furthermore, JunB mRNA expression is down-regulated in many ovarian tumor tissues examined. Thus, the coiled-coil-mediated cooperation between BRCA1 and JunB may facilitate the function of these proteins in tissue-specific transcriptional regulation and tumor suppression.

[*Key Words*: BRCA1; AP1; Jun, transcription; bZIP; coiled-coil]

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Mutations in BRCA1 account for a significant proportion of hereditary breast and ovarian cancers (Welcsh and King 2001). Intense research in the past several years has implicated BRCA1 in regulation of multiple aspects of nuclear function including transcriptional activation, DNA repair, recombination, and checkpoint control (Zhang et al. 1998b; Monteiro 2000; Scully and Livingston 2000; Zheng et al. 2000; Parvin 2001). When tethered to a transcriptional promoter, the BRCA1 carboxyl terminus (BRCT) domain can stimulate transcription and remodel chromatin (Chapman and Verma 1996; Monteiro et al. 1996; Hu et al. 1999). The functional relevance of these studies is underscored by the observation that cancer-predisposing mutations in the same region abolish the activity of the BRCT domain in transcription and chromatin remodeling. Consistent with its potential role in transcriptional regulation, BRCA1 is associated with the RNA polymerase II holoenzyme and chromatin-modifying proteins (Scully et al. 1997; Neish et al. 1998; Yarden and Brody 1999; Bochar et al. 2000; Pao et al. 2000). In addition, the full-length BRCA1 protein can potentiate transcription from several natural promoters (Somasundaram et al. 1997; Ouchi et al. 1998; Zhang et al. 1998a; Harkin et al. 1999; MacLachlan et al. 2000).

In addition to the BRCT domain (AD2), a second *trans*-activation domain of BRCA1 (AD1) was recently discovered (Hu et al. 2000; see Fig. 1A). Moreover, a highly conserved coiled-coil motif in AD1 is critical for its function in transcriptional activation. Interestingly, this coiled-coil region is located immediately upstream of a demarcation point for cancer-predisposing mutations of *BRCA1* in which, according to a phenotype-genotype correlation study, change in ovarian cancer risks occurs (Gayther et al. 1995). Therefore, it has been suggested that this region of the protein may contain a functional domain that specifically protects ovarian epithelial cells from developing tumors (Rahman and Stratton 1998).

In comparison with AD2, transcriptional activation by AD1 is cell-type dependent and less robust (Hu et al. 2000). In some cell lines (e.g., ES2, an ovarian cancer cell line), AD1 by itself exhibits very modest transcriptional activity, but it can synergistically stimulate transcription with AD2, whereas in other cell lines (e.g., HEK293T, an embryonic kidney cell line), AD1 does not confer transcriptional stimulation, either alone or with AD2. The molecular basis for the cell-context dependence of AD1 activity remains to be elucidated, but one likely possibility is that a putative partner(s) that mediates AD1 function may be limiting in these cells.

Herein, we find that AD1 interacts with the Jun proteins of the AP1 family. We show that the BRCA1-Jun interaction is mediated by the coiled-coil region of BRCA1 and the bZIP region of the Jun proteins. Furthermore, the cellular level of JunB is an important determinant for the potency of AD1 in transcriptional activation. We show that the mRNA level of JunB is down-regulated in the majority of ovarian tumor tissues

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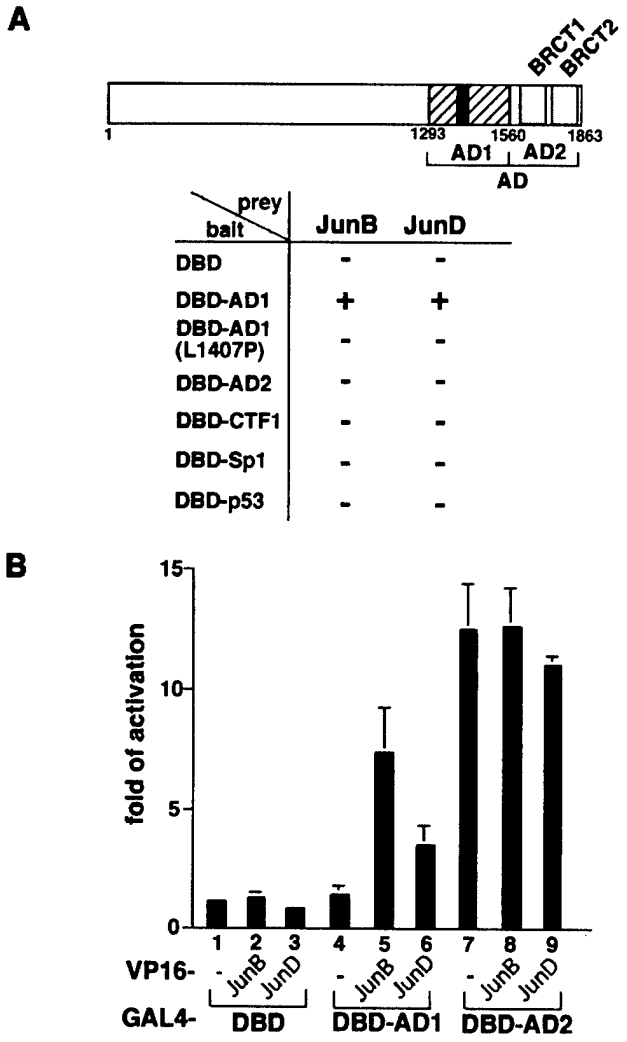

Figure 1. BRCA1 interacts with JunB and JunD in yeast and mammalian two-hybrid assays. (*A*) Summary of the results from the yeast two-hybrid screen. The plus sign (+) indicates rapid growth of the yeast cells in the selective medium as a result of elevated expression of the *HIS3* gene. Also presented is a schematic diagram of the BRCA1 protein, illustrating the location of the *trans*-activation domains. The solid bar within AD1 indicates the coiled-coil motif. (*B*) Mammalian two-hybrid assay showing the interactions between BRCA1 and the Jun proteins. HEK293T cells were transfected with the mammalian bait and prey constructs. Shown in the y axis is the fold of increase in the luciferase activity over the negative control (column 1; GAL4–DBD and VP16).

examined. Thus, the physical and functional link between BRCA1 and JunB may be important for their functions in tissue-specific transcriptional regulation and suppression of tumor development.

Results

JunB and JunD interact with AD1 of BRCA1 in the yeast and mammalian two-hybrid systems

To identify the potential partner(s) of BRCA1 that mediate AD1 function in transcriptional activation, we conducted a yeast two-hybrid screen using AD1 as the bait and a prey library of human ovary cDNA. To reduce the high background due to the intrinsic transcriptional activity of AD1, 100 mM 3-aminotriazole (3-AT) was included in the medium throughout the screen. A large-scale screen uncovered multiple independent cDNA clones that encode human JunB and JunD, two members of the activation protein 1 (AP1) family (Fig. 1A). The AP-1 family of transcription factors consists of homodimers and heterodimers of the Jun and Fos subfamilies that play important roles in diverse aspects of cell proliferation and differentiation (Mechta-Grigoriou et al. 2001; Shaulian and Karin 2001). Mammalian Jun proteins include c-Jun, JunB, and JunD; Fos proteins include c-Fos, FosB, Fra1, and Fra2. A common characteristic of the AP1 family members is the presence of the basic leucine zipper (bZIP) motif that serves as the DNA-binding and dimerization domains (Chinenov and Kerppola 2001).

The interactions between AD1 and the Jun proteins detected in the yeast system were specific, as JunB and JunD failed to bind to other known *trans*-activation domains, including AD2 of BRCA1, and the activation domains of several other mammalian transcription factors such as p53, Sp1, and CTF1 (Fig. 1A). Previous work has shown that a mutation at one of the key leucine residues in the coiled-coil motif in AD1 (L1407P) abolishes its function in transcriptional activation (Hu et al. 2000). The same mutation also abrogated the ability of AD1 to bind to the Jun proteins (Fig. 1A). Interestingly, all partial cDNA clones of JunB and JunD isolated from the screen encode the bZIP domain, suggesting that the AD1-Jun interaction may be mediated by the coiled-coil region of BRCA1 and the bZIP region of the Jun proteins.

To confirm the yeast two-hybrid results, we also used a mammalian two-hybrid assay in which GAL4–AD1 was coexpressed with JunB or JunD that was fused to a potent transcriptional activation domain (VP16). Consistent with previous findings (Hu et al. 2000), GAL4–AD1 alone did not significantly activate transcription in HEK293T cells (Fig. 1B, cf. column 1 with 4), whereas GAL4–AD2 functioned as a potent activator in the same cellular context (Fig. 1B, column 7). Coexpression of VP16–JunB resulted in a significant elevation of transcriptional activation by GAL4–AD1 (Fig. 1B, cf. column 4 with 5). VP16–JunD also had a similar, albeit less pronounced, effect on GAL4–AD1 (Fig. 1B, column 6). In contrast, the same prey constructs did not enhance the activity of GAL4–DBD (Fig. 1B, columns 1–3) or GAL4–AD2 (Fig. 1B, columns 7–9). Expression of the GAL4 derivatives was unaffected by the VP16–Jun proteins (data not shown). Thus, the results from both yeast and mammalian two-hybrid systems indicate that JunB and JunD interact with AD1 in vivo.

BRCA1 specifically interacts with the Jun proteins of the AP1 family

To verify the two-hybrid findings, we examined the ability of various AP1 family members to interact with the

native full-length BRCA1 in human cells. FLAG-tagged c-Jun, JunB, JunD, and c-Fos were expressed in HEK293T or ES2 cells (Fig. 2A, top). Following immunoprecipitation with an anti-FLAG antibody, the presence of the endogenous BRCA1 in the immunoprecipitates was detected by immunoblotting with an anti-BRCA1 antibody (Fig. 2A). Consistent with the two-hybrid results, the FLAG-tagged JunB and JunD were associated with native BRCA1 (Fig. 2A, lanes 2 and 4). In addition, BRCA1 was also coprecipitated with the FLAG-tagged c-Jun (Fig. 2A, lane 3). The in vivo association of BRCA1 and the Jun proteins was unlikely to be mediated by nucleic acids, as it was not affected by the treatment of nuclease or ethidium bromide (data not shown).

In contrast to the Jun proteins, the FLAG-tagged c-Fos did not bind to native BRCA1 in either HEK293T (Fig. 2A, lane 5) or ES2 cells (Fig. 2A, lane 9). For reasons that will become obvious later, the co-IP experiment in HEK293T cells was also repeated in the presence of ectopically expressed HA-JunB. Once again, no endogenous BRCA1 was detected in the FLAG-cFos immunoprecipitate (Fig. 2A, lane 6). It is known that c-Fos forms heterodimers with the Jun proteins, but not homodimers with itself in vivo (Karin et al. 1997). Endogenous Jun proteins were coimmunoprecipitated with the FLAG-cFos (data not shown). Thus, our finding suggests that BRCA1 does not bind to either c-Fos monomers or Jun-Fos heterodimers.

Next, we sought to ascertain the interaction between BRCA1 and the Jun proteins in a more direct manner. The bZIP region of various AP1 proteins was fused with glutathione S-transferase (GST). The purified GST proteins were immobilized on glutathione beads and incubated with the in vitro translated, ^{35}S-labeled AD fragment of BRCA1. As shown in Figure 2B, AD was pulled down by all three GST–Jun fusion proteins (Fig. 2B, lanes 2–4), but not by GST alone (Fig. 2B, lane 1) or GST–cFos (Fig. 2B, lane 5). This result suggests a direct interaction between BRCA1 and the Jun proteins. Furthermore, the in vitro finding confirms that the bZIP region of the Jun proteins is sufficient for binding to BRCA1.

To ascertain the BRCA1–Jun interaction in a more physiological context, the endogenous AP1 proteins from ES2 cells was immunoprecipitated with various commercially available antibodies. Subsequent immunoblotting with an anti-BRCA1 antibody showed that the endogenous BRCA1 was coprecipitated with both c-Jun and JunB, but not c-Fos (Fig. 2C, cf. lanes 2, 4, and 6–8). In addition, the BRCA1 signals were diminished when two antibody-specific competing peptides were included in the immunoprecipitation reactions (Fig. 2C, cf. lane 2 with 3 and 4 with 5). A reciprocal co-IP experiment using two different anti-BRCA1 antibodies also shows the physical association between BRCA1 and c-Jun. Interestingly, the BRCA1–Jun interaction is refractory to a fairly high-salt and detergent concentration (500 mM NaCl and 1% NP-40). Taken together, the results strongly indicate an in vivo association of BRCA1 with specific members of the AP1 family.

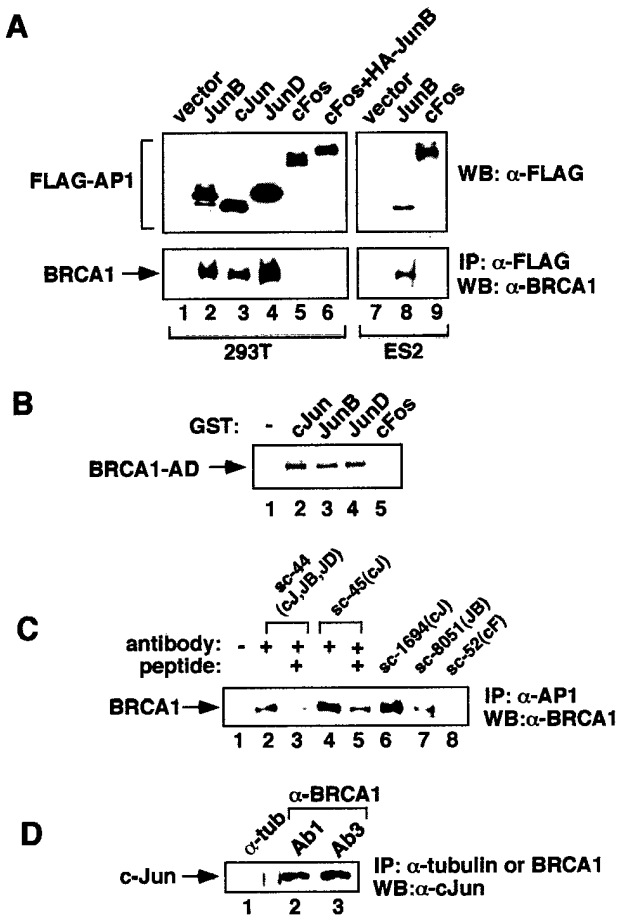

Figure 2. Interaction of the Jun proteins with BRCA1. (*A*) Human HEK293T (lanes *1–6*) or ES2 (lanes *7–9*) cells were transfected with the various expression vectors for the FLAG-tagged AP-1 proteins. Following immunoprecipitation (IP) with an anti-FLAG antibody, the proteins in the immunoprecipitates were resolved by SDS-PAGE, and the presence of the endogenous BRCA1 proteins was detected by immunoblotting (WB) with an anti-BRCA1 antibody (*bottom*). Equal amounts of the cell lysates were analyzed by immunoblotting for the expression of the FLAG-tagged AP-1 proteins (*top*). Lane *6* shows that c-Fos does not interact with BRCA1, even in the presence of exogenous HA-tagged JunB. (*B*) ^{35}S-labeled BRCA1–AD was made with an in vitro translation kit (Promega), and incubated with the GST alone (lane *1*) or GST fused with the bZIP region of various AP1 proteins (lanes *2–5*) that were immobilized on glutathione beads. After extensive washing, the coprecipitated proteins were analyzed by SDS-PAGE and fluorography. (*C*) Coimmunoprecipitation of native BRCA1 and native Jun proteins in ES2 cells. Lysates of ES2 cells were immunoprecipitated with different anti-AP1 antibodies (sc-44 for pan-Jun, sc-45 and sc-1694 for c-Jun, sc-8051 for JunB, and sc-52 for c-Fos). The presence of BRCA1 in the immunoprecipitates was detected by Western blotting using an anti-BRCA1 antibody (Ab-1 from Oncogene). In lanes *3* and *5*, an excess of the corresponding competing peptides was included in the immunoprecipitation reactions. (*D*) A reciprocal co-IP was performed in ES2 cell lysates using either anti-α-tubulin (as a negative control) or anti-BRCA1 antibodies (Ab1 and Ab3; Oncogene) in the immunoprecipitation. The blot was probed with an anti-cJun antibody (sc-1694).

AD1 is necessary and sufficient for BRCA1 binding to the Jun proteins

To further characterize the Jun-binding domain in BRCA1, we constructed a series of deletional mutants and analyzed their ability to interact with the FLAG–JunB using the coimmunoprecipitation assay. The majority of cancer-predisposing mutations in BRCA1 result in truncation of the protein. As summarized in Figure 3, a disease-associated BRCA1 truncation mutant that retained the coiled-coil region in AD1 still interacted with BRCA1 (Fig. 3, construct 2), whereas those that lacked the AD1 region failed to bind to JunB (Fig. 3, constructs 3 and 4). In a different set of mutational analyses, multiple fragments that spanned the entire BRCA1 protein were tested for JunB binding (Fig. 3, constructs 5–12). The only fragments that displayed significant affinity for JunB were those that contained AD1 (Fig. 3, constructs 10 and 12). Further deletional studies within AD1 (Fig. 3, constructs 13–16) revealed a minimal JunB-binding domain (Fig. 3, construct 15; amino acids 1343–1440), which consists of the coiled-coil motif and an ~60 amino acid upstream sequence. This finding indicates that the AD1 region is both necessary and sufficient for BRCA1 binding to JunB.

The coiled-coil motif in BRCA1 is critical for binding to Jun and for AD1-mediated transcriptional activation

To establish a stronger link between the BRCA1–Jun interaction and AD1-dependent transcriptional activation,

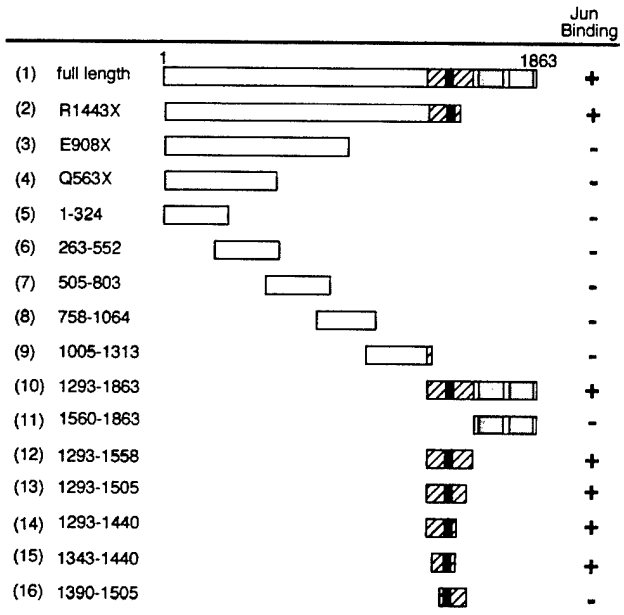

Figure 3. Characterization of the Jun-binding domain in BRCA1. Various HA-tagged BRCA1 fragments were ectopically coexpressed with FLAG–JunB in HEK293T cells. Immunoprecipitation was carried out using an anti-FLAG antibody. The plus sign indicates a significant signal of the BRCA1 fragments in the anti-FLAG immunoprecipitates. The hatched box indicates AD1, whereas the shaded boxes designate the two BRCT repeats in AD2. The solid bar within AD1 represents the coiled-coil region.

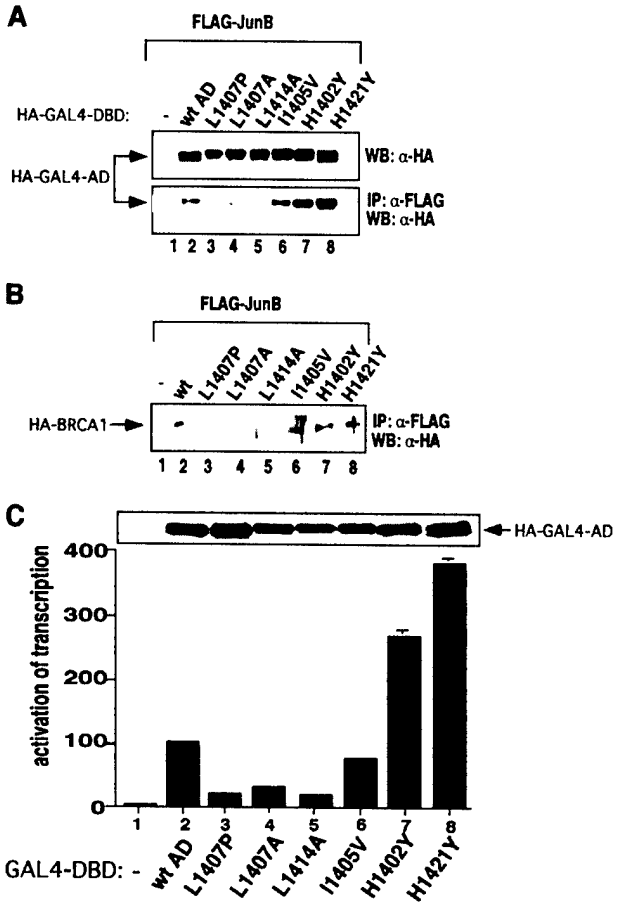

Figure 4. BRCA1-Jun interaction correlates with AD1-mediated transcriptional activation. (*A*) HEK293T cells were cotransfected with the expression vectors for the FLAG-tagged JunB and HA-tagged GAL4–AD domain. Lysates from the transfected cells were immunoprecipitated (IP) with an anti-FLAG antibody, and the immunoprecipitates were probed by immunoblotting (WB) with an anti-HA antibody (*bottom*). As a control, expression of the wild-type and mutant GAL4–AD fusion proteins was detected by immunoblotting of the crude lysates using the anti-HA antibody (*top*). (*B*) HA-tagged full-length BRCA1 proteins were coexpressed with FLAG–JunB in HEK293T cells. Anti-FLAG immunoprecipitation was followed by immunoblotting using the anti-HA antibody. (*C*) ES-2 cells were cotransfected with the GAL4–AD expression vectors and a GAL4-responsive luciferase reporter plasmid. Also shown at *top* of the graph is a Western blot for the wild-type and mutant fusion proteins. The relative transcriptional activity of the wild-type GAL4–AD construct is set at 100.

we introduced various point mutations into the coiled-coil region of AD1 (Fig. 4). All mutants were expressed at similar levels as the wild-type proteins (Fig. 4A,C). The mutational effect on the BRCA1–JunB interaction was tested in the contexts of both AD (Fig. 4A) and the full-length BRCA1 (Fig. 4B). Extensive work on other coiled-coil proteins has shown that the leucine residues at position d of the heptad repeat are the critical determinants for the coiled-coil structure (Lupas et al. 1991; Lupas 1996). Substitution of two such leucines in the coiled-

coil region of BRCA1 (L1407 and L1414) abrogated the BRCA1–JunB interaction (Fig. 4A,B, lanes 3–5). Importantly, the same mutations also impaired the transcriptional activity of GAL4–AD in the luciferase reporter assay (Fig. 4C, lanes 3–5). In contrast, mutations that presumably did not affect the coiled-coil structure (I1405V, H1402Y, and H1421Y) still retained the affinity of BRCA1 for JunB (Fig. 4A,B, lanes 6–8). Likewise, the GAL4 fusion proteins carrying these mutations were still capable of stimulating transcription (Fig. 4C, lanes 6–8). All of the mutations shown in Figure 4 had the same effect on c-Jun and JunD binding as they did on JunB (data not shown). Thus, the affinity of BRCA1 for the Jun proteins strongly correlates with the strength of the *trans*-activation domain in transcriptional stimulation.

JunB potentiates AD1 function in transcriptional activation

Previous characterization of AD1 indicates that this *trans*-activation domain functions in a cell context-dependent manner and that it displays a less robust transcription activity than AD2. For example, HEK293T cells were deficient in AD1-mediated transcriptional activation, despite their ability to support high-transfection efficiency and robust expression of the GAL4 derivatives (Hu et al. 2000). Given the specific interactions between AD1 and the Jun proteins, we speculated that the lack of AD1 function in HEK293T cells might be due to limited expression of one or more Jun proteins. Immunoblotting of crude lysates from HEK293T cells indicated that the protein level of JunB was extremely low (Fig. 5A, lane 2), as has been observed by others (Bakiri et al. 2000). In contrast, the levels for c-Jun, JunD, and c-Fos in HEK293T cells were comparable with those in the other cell lines examined (Fig. 5A).

To determine whether the low level of JunB protein in HEK293T cells was causally related to the lack of AD1 activity, we asked whether the deficiency in supporting AD1 function could be rescued by ectopic expression of JunB. As shown in Figure 5B, coexpression of FLAG–JunB and GAL4–AD1 significantly enhanced the ability of AD1 to activate transcription (Fig. 5B, cf. lanes 6 and 7). However, JunB did not superactivate GAL4–AD2 (Fig. 5B, cf. lanes 11 and 12), nor did it rescue the transcriptional defect of a coiled-coil mutant of AD1 that failed to bind JunB (L1407P; Fig. 5B, cf. lanes 16 and 17). These results strongly indicate that JunB potentiates BRCA1 function through its interaction with the coiled-coil region of AD1. In contrast to JunB, ectopic expression of c-Jun, JunD, or c-Fos failed to complement the deficiency of HEK293T cells (Fig. 5B, lanes 8–10), despite their expression levels equivalent to that of junB (Fig. 2A). This differential effect of the AP1 proteins was observed at multiple concentrations of the AP1 expression vectors (data not shown). Thus, although all three Jun proteins are capable of binding to BRCA1, JunB exhibits a distinct function in facilitating AD1-mediated transcriptional activation.

Although JunB protein was expressed above the detectable level in most of the cell lines examined (Fig. 5A), it could still be limiting for supporting AD1-mediated transcriptional activation. For example, despite the higher JunB expression in ES2 cells than that in HEK293T cells, AD1 alone only moderately activates transcription in ES2 cells (Hu et al. 2000) (Fig. 5C, cf. lanes 1 and 6). Ectopic expression of JunB, and JunD to a lesser extent, enhanced AD1-mediated transcriptional activation in ES2 cells (Fig. 5C, columns 7 and 9). As observed in HEK293T cells, c-Jun and c-Fos failed to confer such superactivation of AD1 (Fig. 5C, columns 8 and 10). Therefore, our data indicate that the strength of AD1 in transcriptional activation is limited by the cellular level of JunB. An interesting difference between HEK293T and ES2 cells with regard to AD1 function is that ES2, but not HEK293T cells, can support a synergistic action of AD1 and AD2 (Hu et al. 2000). It is therefore plausible that the level of JunB in ES2 cells, although insufficient for supporting maximal activation by AD1 alone, may be replete for the cooperative activation by AD1 and AD2.

Given the proximity of the Jun-binding domain to the previously defined transition point of *BRCA1* mutations that are associated with higher ovarian cancer risk (Gayther et al. 1995), we speculated that this domain likely provides special protective functions against ovarian cancer. In such an event, a deficit of JunB in ovarian epithelium might contribute to development of ovarian cancer in particular. To test this possibility, we compared the JunB mRNA level in tumor and normal tissues from the same individuals. The normalized real-time PCR results are shown in Figure 5D. In seven of the nine matched cDNA pairs from ovary tissue, JunB expression is significantly lower in tumor than in the normal issues (Fig. 5D, pairs 1–5, 7, and 8). The remaining two ovarian pairs (Fig. 5D, pairs 6 and 9) had very low JunB mRNA levels even in the normal tissues, suggesting that there might be some intrinsic abnormality in these two normal cases. Interestingly, the differential expression of JunB in most ovarian pairs was not obvious in a panel of matched cDNA pairs from breast tissue (Fig. 5D, pairs 10–16). This finding is consistent with the notion that the BRCA1–JunB interaction may play a role in specific suppression of ovarian cancer development.

Discussion

A wealth of evidence strongly suggests that BRCA1 plays an important role in the maintenance of genome stability via its function in transcriptional regulation and DNA repair. However, it remains puzzling that disease-associated mutations in *BRCA1*, which compromise such universal nuclear functions as transcription and DNA repair, specifically lead to elevation of the risk in developing breast and ovarian cancers. It has been suggested that the rapid proliferating status of the breast epithelium during puberty could render it particularly susceptible to *BRCA1* mutation-dependent tumorigenesis (Scully and Livingston 2000). In addition, the fact that breast and ovary are both estrogen-responsive tissues and that BRCA1 can modulate transcriptional activation by estrogen receptor could also

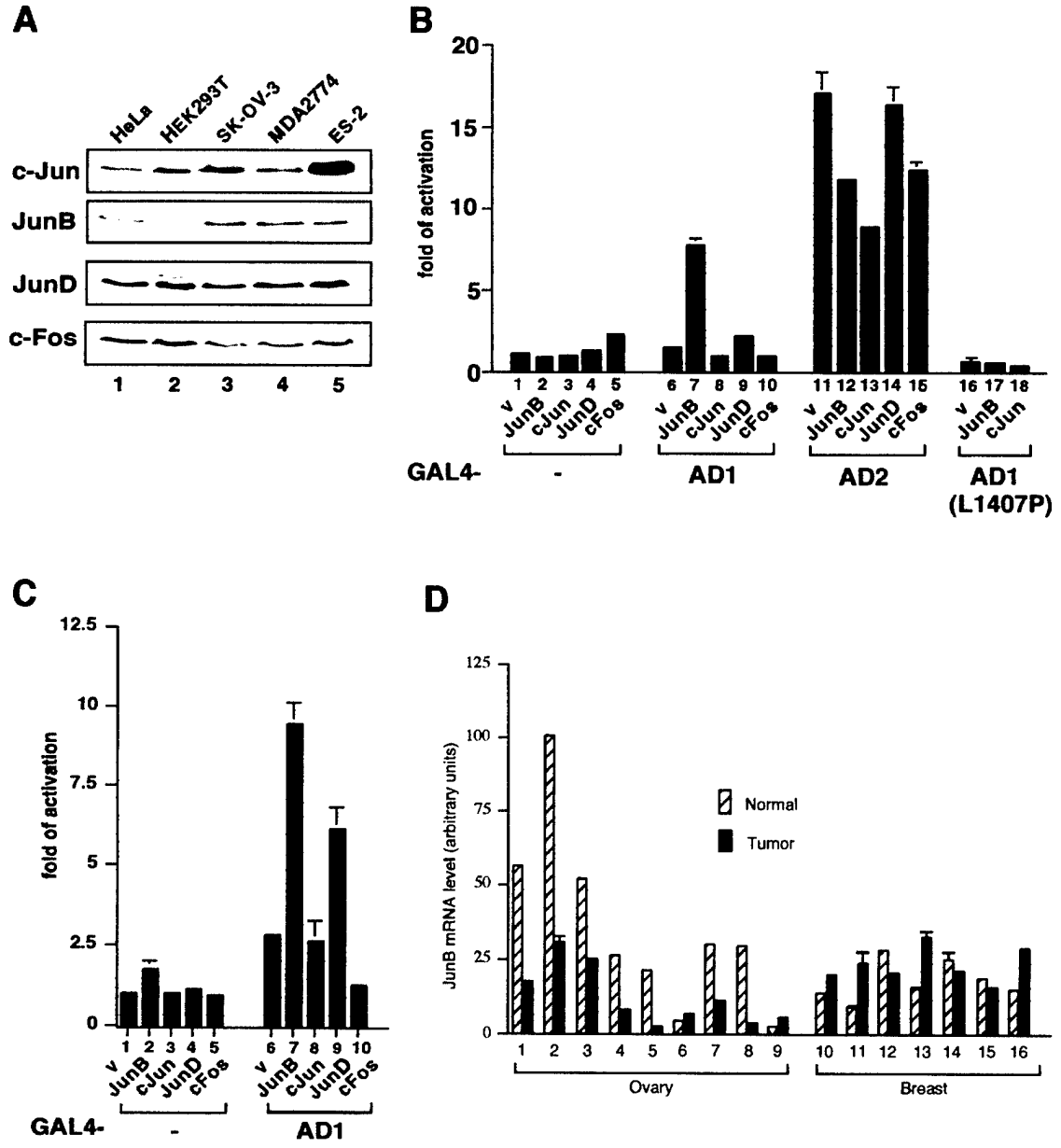

Figure 5. JunB can superactivate AD1-dependent transcriptional activation. (*A*) An equal amount of cell lysates from various cell lines was probed by immunoblotting with specific antibodies against c-Jun, JunB, JunD, and c-Fos. (*B*) HEK293T cells were cotransfected with the luciferase reporter plasmid, the GAL4 derivatives, and various AP1 expression vectors. The relative luciferase activity in the presence of GAL4–DBD alone (column 1) is set at one. (*C*) Luciferase reporter assay was performed in ES2 cells in the same manner as described in *B*. (*D*) Normalized matched cDNA pairs of normal and tumor ovarian (*1–9*) or breast (*10–16*) tissues were analyzed by the real-time PCR reactions for JunB mRNA expression.

explain the organ-specific nature of the BRCA1-dependent cancer risk (Fan et al. 1999, 2001; Zheng et al. 2001). It is also possible that the tissue-specific action of BRCA1 may be determined by more than one BRCA1-associated protein complex. In such an event, changes in the level and/or biochemical properties of a number of the BRCA1-associated proteins could contribute to the development of neoplasm in these tissues.

The findings in the current study strongly suggest that JunB plays an important role in mediating the function of one of the *trans*-activation domains of BRCA1 (AD1). First, both in vitro and in vivo experiments indicate that the two proteins interact through the coiled-coil domain of BRCA1 and the bZIP domain in JunB. The affinity of BRCA1 for JunB is strongly correlated with the strength of the AD1 domain in transcriptional activation. Furthermore, the data suggest that the cellular level of the JunB protein is an important determinant for AD1 function in transcriptional activation. Limited AD1 transcriptional activity due to a deficit of JunB can be rescued

by ectopic expression of JunB. This finding provides a molecular explanation for the previously observed cell-type dependent behavior of AD1 (Hu et al. 2000). Lastly, JunB mRNA levels are down-regulated in many ovarian tumor tissues compared with the normal tissues from the same individuals. Given that the AD1 domain of BRCA1 encompasses the demarcation point for *BRCA1* mutations that are associated with increased risk of ovarian cancer, our results raise an intriguing possibility that the BRCA1–JunB interaction may be particularly important for suppression of ovarian cancer. However, due to the lack of detailed information concerning the clinical characteristics of the patients and the exact cell types from which the cDNA pairs were prepared, further study is needed to explore the exact biological significance of the differential JunB expression observed in the current work.

The exact geometry and stoichiometry of the BRCA1–Jun complexes remain to be determined. It is also possible that the BRCA1–Jun interaction is aided by additional factors. The finding that BRCA1 is not associated with the cFos-containing complexes strongly suggests that Jun-Fos dimers, which is the predominant heterodimeric form of the AP1 family in vivo, are not capable of binding to BRCA1. This raises the possibility that BRCA1 may specifically target Jun monomers and/or Jun–Jun dimers. Conceivably, this Jun-specific interaction may lead to changes in the relative abundance, subnuclear localization, and biochemical characteristics of various forms of the AP1 proteins. The bZIP sequences among the Jun proteins are highly conserved, whereas those of Fos and Jun are relatively divergent. This could explain the disparity in their binding affinity for BRCA1. It is tempting to speculate that the leucine zipper motif may only provide the architectural basis for binding to BRCA1. Additional amino acid residues in the bZIP region that are unique to the Jun proteins may serve as the actual contact points for the coiled-coil region of BRCA1.

Whereas all three Jun proteins are capable of interacting with BRCA1, only JunB exhibits a strong enhancing effect on the AD1 transcriptional activity. Such a functional difference among the Jun proteins could be attributed to the more divergent sequences outside of the bZIP domain. The observation that BRCA1 can selectively target specific members of the AP1 family for physical and functional interaction may have profound biological ramifications. AP-1 family members form a large number of homodimers and heterodimers in vivo, each of which may exhibit distinct regulatory properties (Karin et al. 1997). As a consequence, different AP1 family members can play diverse and even opposing roles in cell proliferation and differentiation. For example, it has been well documented that c-Jun is positively involved in cell proliferation and Ras-mediated oncogenesis (Mechta-Grigoriou et al. 2001; Shaulian and Karin 2001). On the other hand, JunB and JunD can suppress Ras- and Src-induced cellular transformation (Johnson et al. 1996; Mechta et al. 1997). Moreover, JunB can antagonize the stimulatory function of c-Jun in cyclin D1 transcription (Bakiri et al. 2000), and at the same time activate the transcription of $p16^{INK4a}$, an inhibitor of the cyclin D-CDK4–D-CDK6 complexes (Passegue and Wagner 2000). Recent work with conditional JunB knockout mice also supports a role of JunB in tumor suppression (Passegue et al. 2001). Thus, although our study does not exclude a potential functional link between BRCA1 and the other two Jun proteins, the cooperation between BRCA1 and JunB in transcriptional regulation may be related to their known functions in suppression of tissue-specific tumor development.

Materials and methods

Yeast two-hybrid screen

The standard yeast two-hybrid screen was performed in the following manner. First, bait plasmid was generated by inserting a PCR-amplified cDNA fragment encoding AD1 (amino acids 1293–1559) of BRCA1 into pGBT8 (Clontech), resulting in an in-frame fusion with the GAL4 DNA-binding domain (DBD). Second, the resultant plasmid, pGBT8-AD1, and a human ovary cDNA library (Clontech) were cotransformed into the *Saccharomyces cerevisiae* reporter strain Hf7C according to the manufacturer's instructions (Clontech). Transformants were plated on synthetic medium lacking tryptophan, leucine, and histidine, but containing 100 mM 3-aminotriazole (3-AT), which was used to suppress the relatively high background due to the intrinsic transcripional activity of the bait construct. Approximately 26 million transformants were screened, of which 32 were judged to be strongly HIS-positive. Additional screens using a number of negative controls were carried out (see Fig. 1A), and only those candidates exhibiting AD1-specific interaction were characterized further. Twenty clones contained partial cDNA sequences for JunD, and six contained the JunB cDNA sequences.

Mammalian cell transfection and luciferase assay

HEK293T cells were maintained in DMEM medium with 10% fetal calf serum and were transfected using LipofectAmine 2000 (GIBCO BRL). ES-2 cells were grown in McCoy's 5A medium with 10% fetal calf serum and were transfected using LipofectAmine Plus (GIBCO BRL). In a typical GAL4-based transcription reporter assay performed in HEK293T cells, 0.5 μg reporter and 1.0 μg protein expression vectors were used. Half of these amounts were used for the luciferase assays in ES2 cells. The luciferase assays were performed as described previously (Hu et al. 2000). The expression vectors for GAL4 derivatives used in the mammalian two-hybrid assay were described previously (Hu et al. 2000). The vectors for the VP16 fusion proteins used in the mammalian two-hybrid assay were constructed by cloning the cDNA sequences for JunB and JunD into the prey plasmid as described previously (Yu et al. 1998).

Coimmunoprecipitation

The FLAG-tagged AP1 proteins were expressed from the pcDNA3 vector (Clontech). Plasmids pCG-HA-GAL4(1–94)–AD (Hu et al. 2000) and pcDNA3β-HA-BRCA1 (full length) (Scully et al. 1997) were described previously. Twenty-four hours after transfection, cells were washed twice with PBS and lysed in 0.5 mL Lysis Buffer (50 mM HEPES at pH 8.0, 250 mM NaCl, 0.1%NP-40, and protease inhibitor tablets from Roche-

Bohringer). After brief sonication, the lysate was centrifuged at 14,000 rpm for 12 min at 4°C. The supernatant was used for subsequent coimmunoprecipitation. Fifteen microliters of 50% slurry of the anti-FLAG agarose beads (Sigma) were used in each immunoprecipitation, and the tubes were rotated overnight at 4°C. Following incubation, the beads were centrifuged at 3300 rpm for 2 min, and washed once with the lysis buffer and twice with a washing buffer (50 mM HEPES at pH 8.0, 500 mM NaCl, 1% NP-40), with each wash lasting at least 1 h. The precipitates were then eluted in 10 µL 2× protein sample buffer and loaded on SDS–polyacrylamide, followed by Western blotting according to the standard procedures. Five microliters of the input crude extract were used for detecting protein expression levels. The presence of the endogenous BRCA1 was detected using a commercially available anti-BRCA1 antibody (Ab-1 from Oncogene). The HA-tagged proteins were detected using an anti-HA monoclonal antibody (12CA5).

The coimmunoprecipitation experiment shown in Figure 2C was conducted using lysates from ES2 cells, anti-Jun antibodies for immunoprecipitation, and an anti-BRCA1 antibody (Ab-1 from Oncogene) for immunoblotting. A total of 1.5 µg of the following commercially available anti-AP1 antibodies (Santa Cruz Biotech.) were used in immunoprecipitation: sc-44 (anti-cJun, JunB, and JunD); sc-45 (anti-cJun); sc-1694 (anti-cJun); sc-8051 (anti-JunB); sc-52 (anti-cFos). Two competing peptides for c-Jun in immunoprecipitation (sc-44p and sc-52p; Santa Cruz Biotech.) were used. The reciprocal co-IP shown in Figure 2D was done using 2.5 µg of anti-BRCA1 (Ab1 and Ab3) or anti-α-tubulin antibodies (Ab-1; Oncogene) for IP and an anti-cJun antibody (sc-1694) for immunoblotting.

In vitro GST pulldown assay

The PCR fragments encoding the bZIP region of the AP1 proteins were fused in-frame with the GST portion in plasmid pGEX-2T (Pharmacia). The GST–bZIP proteins were expressed and purified according to the manufacturer's instruction, with the induction of the protein expression performed at 37°C for 3 h. The fusion gene encoding FLAG-tagged BRCA1–AD in the pcDNA3 vector was also under the control of the bacteriophage T7 promoter. This plasmid was used for in vitro transcription and translation in the TnT Reticulocyte Lysate system (Promega). The ^{35}S-labeled FLAG–BRCA1–AD was mixed with 10 µg of GST derivatives bound to agarose beads in 0.5 mL of binding buffer (50 mM HEPES at pH 8.0, 150 mM NaCl, 1 mM EDTA, 0.1% NP-40 and protease inhibitor tablets). The binding reaction was carried out at 4°C overnight and subsequently washed four times with the washing buffer (the same as binding buffer, except 0.5% NP-40), 30 min each round. The beads were eluted in 10 µL of 2× protein sample buffer and the proteins were resolved on 10% SDS–polyacrylamide. The gel was then dried and exposed to X-ray films overnight.

Real-time PCR

The ABI PRISM 7700 Sequence Detection System (PE Biosystems) was used for the quantitative analysis of JunB mRNA expression. The following primers were designed using the Primer Express software provided by the manufacturer. JunB forward primer, ACTCATACACAGCTACGGGATACG; JunB reverse primer, GGCTCGGTTTCAGGAGTTTG; TagMan probe, (VIC)CCCCTGGTGGCCTCTCTCTACACGATA(MRA).

Primers, probes, and TagMan universal PCR master mixes were purchased from Applied Biosystems. PCR was performed in a 50-µL reaction volume, using the default assay condition in the ABI PRISM 7700 Sequence Detector. The final concentrations for primers and probe were 300 and 250 nM, respectively. cDNA pairs (Clontech) normalized with β-actin and/or ribosomal protein S9 were used as the templates in the PCR reactions. In Figure 5D, pairs 1–4 come from HP101O, HP103O, HP104O, and HP105O; pairs 5–9 from a human ovary-matched cDNA pair panel (K1435-1); pairs 10–11 from HB102B and HP104B; and pairs 12–16 from a human breast-matched cDNA pair panel (K1432-1).

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