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 domain at the C terminus (see Fig. 1A). 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...
various lengths of the BRCA1 C-terminal region were amplified by a BRCA1 cDNA clone by a standard PCR method. The PCR fragments were subsequently cloned into the NheI and BamHI 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 NheI and XhoI 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 XhoI 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 XhoI and BamHI 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 pcDNA3βHA/BRC1 (16). The yeast strains HFC7 (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. 1B; 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. 1B). 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. 1B).
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. 1B). 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. 1A).

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. 1C). 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. 1C), despite the fact that HA-NLS-AD was expressed less abundantly than the two smaller fusion proteins (Fig. 1D). 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. 2A 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. 2B). The most striking synergism was observed in HCT116, a colorectal carcinoma cell line. As shown in columns 9–12 of Fig. 2B, 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. 2A).

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.
tested in our study, HEK293T cells displayed the most dramatic difference between AD1 and AD2 activities (columns 3 and 4, Fig. 2C), despite the fact that GAL4-AD1 was more abundant than GAL4-AD2 in these cells (lanes 3 and 4 in Fig. 2A).

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. 2D). For instance, AD1 and AD2 can individually activate transcription in MDAH2774 cells (columns 3 and 4, Fig. 2D). 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. 2D). The three GAL4-derived activators were expressed at a comparable level in these two cell lines (lanes 10–12 in Fig. 2A 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. 3A) and analyzed in both ES-2 and SKOV3 cells. All fusion proteins were expressed at similar levels in each cell line (Fig. 3B 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. 3C) 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. 3C) 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. 3C).

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. 4A, 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 homologs of different species (Fig. 4A). 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. 3C, 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 Ser1423 and Ser1524 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. 3C, 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.
at Ser1423 and Ser1524 indicate the ATM phosphorylation sites.

Asterisks critical determinants for the coiled-coil motif, are highlighted heptad repeats. The hydrophobic coiled-coil sequence (aa 1391–1424) indicate amino acid residues of the B

numbers in structure prediction program (Ref. 36; data not shown).

A similar result was obtained using a different secondary structure prediction program (Ref. 36; data not shown).

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 transactivation domains, the originally identified activation domain possesses only a modest transcriptional activity in vivo (Ref. 22 and this study; see Fig. 1B). Our work in yeast and human cells has led to the discovery of a new activation domain (AD1) in BRCA1 that can function cooperatively 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. 2C). In contrast, both activation domains were equally active in MDAH2774 (Fig. 2D). 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. 2D, 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 sp-

\(^2\) 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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