BRCA1, a breast and ovarian cancer susceptibility gene, encodes a 220-kDa protein whose precise biochemical function remains unclear. BRCA1 contains an N-terminal RING finger that mediates protein-protein interaction. The C-terminal domain of BRCA1 (BRCT) can activate transcription and interacts with RNA polymerase holoenzyme. Using the yeast two-hybrid system, we identified an interaction between the BRCA1 RING finger and ATF1, a member of the cAMP response element-binding protein/activating transcription factor (CREB/ATF) family. We demonstrate that BRCA1 and ATF1 can physically associate in vitro, in yeast, and in human cells. BRCA1 stimulated transcription from a cAMP response element reporter gene in transient transfections. BRCA1 also stimulated transcription from a natural promoter, that of tumor necrosis factor-α, in a manner dependent on the integrity of the cAMP response element. These results implicate BRCA1 in transcriptional activation of ATF1 target genes, some of which are involved in the transcriptional response to DNA damage.

The BRCA1 gene encodes a nuclear protein of 1863 amino acids and fulfills genetic criteria for a tumor suppressor gene in breast and ovarian cancer (1). Mutations in BRCA1 account for a significant proportion of hereditary breast cancer, which represents 5–10% of total breast cancer cases. Although BRCA1 is not mutated in sporadic breast cancer, recent reports suggest that it may be present at reduced levels in high-grade breast carcinoma (2). Although the majority of mutations in BRCA1 result in translation frameshift and premature truncation, several recurring missense mutations were identified. Two of these missense mutations disrupt critical cysteine residues in the BRCA1 RING finger. This observation prompted us to search for proteins capable of interacting with the BRCA1 RING domain.

RING fingers are ~60-amino acid-long motifs with a conserved C3HC4 structure that coordinates the binding of two zinc atoms (4). RING motifs are found in >80 proteins and are thought to mediate protein-protein interactions via a structure that is unique from other zinc fingers (5, 6). The BRCA1 RING finger encompasses residues 1–64 and is virtually identical in the human, mouse, and rat BRCA1 homologues (7). BARD1, a RING finger-containing protein, was shown to interact with the BRCA1 RING finger, yet the functional significance of this interaction is not yet understood (8). Missense mutations of cysteine residues in the BRCA1 RING finger indicate that this domain is likely to be vital for the function of the BRCA1 protein (7). The missense mutant C61G is among the most commonly identified mutations in BRCA1 and was shown to disrupt zinc binding and to interfere with homodimerization in vitro (9).

Several lines of evidence strongly suggest that BRCA1 is involved in transcription (reviewed in Ref. 10). We and others have observed that the C terminus of BRCA1 activates transcription of a reporter gene when fused to a heterologous DNA-binding domain in yeast and mammalian cells (11–13). Tumor-derived missense mutations in the C terminus are defective for transcriptional activation, which indicates that this may be a relevant function for BRCA1. Furthermore, BRCA1 was shown to interact directly with RNA helicase A, a component of RNA polymerase II holoenzyme, via its C-terminal domain (BRCT) (14, 15) and with the p300 coactivator through the C-terminal domain as well as N-terminal domains (16). We observed that overexpression of wild-type BRCA1, but not tumor-derived mutants, can trigger a G1 arrest that is mediated by transcriptional activation of p21Waf1/Cip1 (17). In addition to its association with holoenzyme, BRCA1 can bind to several different transcription factors, including p53, Myc, STAT1, and the CBP-interacting protein p300 (18–22).

The spatial distribution and post-translational state of BRCA1 are regulated in response to DNA-damaging agents. BRCA1 was shown to interact with human RAD51, one of several mammalian homologues of bacterial RecA (23). Human RAD51 can mediate ATP-dependent DNA strand exchange reactions in recombination and DNA repair (reviewed in Ref. 24). BRCA1 colocalizes with human RAD51 during S phase in discrete subnuclear foci of mitotic cells and on synaptonemal complexes of cells undergoing meiosis. In response to DNA-damaging agents, including UV, ionizing radiation, and hydroxyurea, BRCA1 subnuclear localization is altered and found...
to overlap with proliferating cell nuclear antigen-containing nuclear foci at structures undergoing DNA replication (25). These changes correspond with an increase in apparent BRCA1 molecular mass due to phosphorylation. This was recently found to be due to the Chk2 kinase that also regulates p53 activity (26).

The modification of BRCA1 by a cell cycle checkpoint kinase adds to accumulating data that implicate BRCA1 in the maintenance of genome integrity. BRCA1−/− embryonic stem (ES) cells were shown to have an impaired ability to perform transcription-coupled DNA repair (TCR) of oxidative damage induced by ionizing radiation or H2O2 (27). Nullizygous ES cells show a specific defect in TCR. Although BRCA1 was shown to be phosphorylated and relocalized within the nucleus after UV treatment (25), BRCA1−/− ES cells were not impaired in their ability to perform TCR after UV exposure. This suggests that BRCA1 may be directly involved in TCR or that BRCA1 may regulate genes necessary for TCR. Further experiments in BRCA1−/− ES cells and cell lines have supported a role for BRCA1 in DNA repair of double-stranded DNA breaks (28, 29) and found that reinsertion of BRCA1 into cell lines can rescue the response to DNA damage (30, 31). A complex between BRCA1 and BRCA2, the second breast cancer susceptibility gene product, was identified (32). Evidence suggests that BRCA2 is actively involved in ensuring fidelity of double-stranded DNA break repair (33, 34). Taken together, these interactions implicate BRCA1 as part of a macromolecular complex including human RAD51 and BRCA2 that plays a role in DNA repair.

Here we report a specific physical association between the BRCA1 RING domain and ATF1, a member of the CREB/ATF family of basic zipper transcription factors. We demonstrate that BRCA1 is capable of modulating the activity of a reporter gene for CREB/ATF family members. Expression of BRCA1 stimulates the activity of a natural promoter containing a CREB/ATF response element, but fails to stimulate the promoter with a mutated CREB/ATF response element. ATF1 has been implicated as part of a signal transduction pathway that responds to UV damage. These data suggest that BRCA1 is a transcriptional coactivator that can modulate the activity of ATF1. This observation strengthens the hypothesis that BRCA1 has a role in both transcription and DNA damage response.

MATERIALS AND METHODS

**Yeast Two-hybrid Screen**—The Gal4 Matchmaker II system (CLONTECH) was used according to the manufacturer's recommendations. Yeast strain CG1945 was cotransformed with pAS2-RING and a human thymus cDNA library (CLONTECH). Transformants were plated on selective medium with 15 mM 3-aminotriazole, which sets a high stringency for interaction. A total of 37 His+ β-gal− clones were identified, and plasmid DNA was isolated from 23 out of the 37 clones and subjected to DNA sequencing at the Gal4-binding domain junction. Yeast strains were not impaired in their ability to perform TCR after UV exposure. This suggests that BRCA1 may be directly involved in TCR or that BRCA1 may regulate genes necessary for TCR. Further experiments in BRCA1−/− ES cells and cell lines have supported a role for BRCA1 in DNA repair of double-stranded DNA breaks (28, 29) and found that reinsertion of BRCA1 into cell lines can rescue the response to DNA damage (30, 31). A complex between BRCA1 and BRCA2, the second breast cancer susceptibility gene product, was identified (32). Evidence suggests that BRCA2 is actively involved in ensuring fidelity of double-stranded DNA break repair (33, 34). Taken together, these interactions implicate BRCA1 as part of a macromolecular complex including human RAD51 and BRCA2 that plays a role in DNA repair.

**Plasmid Construction**—BRCA1 expression vectors were previously described (17). For immunoprecipitation experiments, a pEF-BOS vector (36) containing full-length human BRCA1 (a gift of Toru Ouchi, Mount Sinai School of Medicine) was used. Full-length human ATF1 was present in clone 31 isolated from the two-hybrid screen described above. ATF1−/− mice (21) was also amplified by polymerase chain reaction (in vitro) using the N-terminal primer 5′-GCGGATCTCATGAAAGATTCCCAAG-3′ and the C-terminal primer 5′-TCTTAGTTATGGGAGGCGGATCATC-3′ recloned into pCRI (Invitrogen), which served as the plasmid template for coupled *in vitro* transcription/translation (Promega). ATP1 deletion mutants were constructed by polymerase chain reaction using the following primers paired with the above primers: ATF1−/− (73–271), N-terminal primer 5′-CCGAAACTCTTGGAGATAGG-3′.
that developed within 1 h; 2 gal, Sigma) as a substrate.

2

Table I

Clone 31 shows a positive interaction with the wild-type but not mutant BRCA1 RING finger

| Bait vector | Prey vector | β-Galactosidase expression |
|-------------|-------------|---------------------------|
| pAS2-1      | Clone 31    | –                         |
| pAS2-RING   | Clone 31    | ++                        |
| pAS2-RING-C61G | Clone 31 | –                         |
| pAS2-RING-C64G | Clone 31 | –                         |
| pLAM5*      | Clone 31    | –                         |
| pVA3        | pTΔ1        | +++                       |

CAGCgggG-C-3’; and ATF1-(1–215). C-terminal primer 5’T-AgtTCtgt-9’ was used as a bait to screen a human thymus cDNA library in yeast strain CG1945. One of the 37 His-β-gal fusions identified was a gift of Nic Jones (Imperial Cancer Research Fund, London).

RESULTS

ATF1 Identified in Yeast Two-Hybrid Screen as a BRCA1-interacting Protein—In an effort to identify BRCA1 partner proteins, we cloned the N-terminal 101 residues of BRCA1 into pAS2-1 (CLONTECH). The resulting vector, pAS2-RING, was used as a bait to screen a human thymus cDNA library in yeast strain CG1945. One of the 37 His-β-gal clones we identified (clone 31) encoded ATF1, a member of the CREB/ATF family.

After plasmid isolation, we recloned the pGAD424 vector (Invitrogen) for in vitro translation. The BamHI/EcoRI fragment encoding full-length human ATF1 was subsequently recloned into pGEX5X-1 (Amersham Pharmacia Biotech) as a GST fusion construct and into pGAD424 for expression studies. All constructs were sequenced to verify correct reading frames for fusion proteins, and protein expression was verified by immunoblot analysis. The Gal4-(1–147)-ATF1 fusion construct was made by amplifying the ATF1 sequences encoding the Gal4 activation domain, a few amino acids, and a stop codon. This was followed by the 5'-untranslated region of ATF1. The start codon, and the entire open reading frame for ATF1. We hypothesized that clone 31 directed the expression of ATF1 from a cryptic promoter and that this expression was responsible for the positive interaction we observed. Efforts to detect the expression of ATF1 from the original plasmid (clone 31) were hampered by poor specificity with two commercial anti-ATF1 antibodies. We note that other groups have identified CREB from two-hybrid screens as a cDNA insert both out of frame and backwards with respect to the Gal4 activation domain (38). Therefore, to determine whether ATF1 could interact with the BRCA1 RING domain in yeast, we recloned ATF1 into pGAD424 as an in-frame fusion protein with the Gal4 activation domain. This plasmid was sequenced to verify the correct reading frame and was used to test for a specific interaction with pAS2-RING. This plasmid, designated pGAD-ATF1, was used to test for a specific interaction with pAS2-RING in subsequent experiments.

The combination of pAS2-RING with pGAD-ATF1 yielded expression of the β-galactosidase reporter gene in yeast (Fig. 1A). Neither plasmid alone was sufficient to activate the reporter gene. We again tested the ability of the two missense mutations in the RING domain (C61G and C64G) to interact with pGAD-ATF1. Yeast transformed with either pAS2-RING-C61G or pAS2-RING-C64G and pGAD-ATF1 showed marked reduction in the expression of β-galactosidase (Fig. 1A) despite comparable levels of expression of wild-type and mutant Gal4-BRCA1 fusion proteins (Fig. 1B). This was identical to the pattern observed for the original clone 31. Thus, we observed a specific interaction between full-length ATF1 and the BRCA1 RING domain in the yeast two-hybrid system. In yeast, the BRCA1 RING domain was sufficient to promote an interaction with ATF1, which was abrogated by either of the two tumor-derived mutations, C61G or C64G.

We confirmed the interaction between the BRCA1 RING domain and ATF1 using a GST pull-down assay. GST fusion proteins containing BRCA1 residues 1–101 or 1–304 were constructed, expressed in E. coli, and purified on glutathione-agarose beads. GST-BRCA1 fusion proteins (Fig. 1B) were purified on glutathione-agarose beads, whereas GST-ATF1 captured radiolabeled BRCA1 to a greater extent than GST alone, yet the interaction appeared to have a relatively low affinity in vitro (6-fold increase above GST alone by densitometric analysis) (Fig. 2B).

To localize the region of interaction between the two proteins, we made two deletion mutants of ATF1. The first mutant, ATF1-(73–271), lacks the first 72 amino acids of ATF and therefore the activation and CBP-binding domains. The second mutant, ATF1-(1–215), lacks the C-terminal basic zipper DNA-binding region. ATF1-(1–215) did not demonstrate appreciable binding to GST-BRCA1-(1–304), whereas ATF1-(73–271) still bound to the BRCA1 affinity reagent (Fig. 2C). This indicates that the BRCA1-ATF1 interaction is specific and is dependent on the RING finger of BRCA1 and the DNA-binding domain of ATF1.

Full-length BRCA1 Interacts with ATF1 in Mammalian Cells—To determine whether BRCA1 could interact with ATF1 in human cells, we expressed full-length BRCA1 in human 293T cells and immunoprecipitated a 220-kDa species with rabbit polyclonal antisera. ATF1 coprecipitated with BRCA1 from cells cotransfected with both BRCA1 and ATF1 expression vectors (Fig. 3A). In 293T cell lysates, the coprecipitation of ATF1 with BRCA1 occurred only when both proteins were overexpressed (Fig. 3A). The most important indication of the physiological significance of the BRCA1-ATF1 interaction was the demonstration of an endogenous complex between BRCA1 and ATF1. Lysates from rapidly growing human MCF-7 breast cancer cells were subjected to immunoprecipitation with a monoclonal antibody directed against the C terminus of BRCA1 (AB3), followed by immunoblotting with an anti-ATF1 antibody. ATF1 was identified by immunoblotting analysis as a 38-kDa species present in BRCA1 immunoprecipitates (Fig. 3B). We also observed that AB1, a monoclonal antibody directed against the N terminus of BRCA1, was incapable of coprecipitating...
ATF1 and in fact might have blocked the interaction between BRCA1 and ATF1. Immunoprecipitation with the combination of AB1 and AB3 (Fig. 3B, third lane) resulted in a reduction of coprecipitating ATF1, supporting the notion that an anti-BRCA1 monoclonal antibody directed against the RING finger can block the interaction between BRCA1 and ATF1.

**BRCA1 Modulates Transactivation of Gal4-ATF1 and Gal4-CREB Fusion Proteins**—Because BRCA1 was shown to regulate transcription (17) and the activity of specific transcription factors (18–21), we sought to determine whether BRCA1 could modulate the transcriptional activity of ATF1 or CREB. We constructed a Gal4-ATF1 fusion protein, which activated a luciferase reporter gene with Gal4 DNA-binding sites (G5-Luc) in the presence of forskolin, an adenylate cyclase agonist, and activator of protein kinase A. Expression of Gal4-ATF1 or Gal4-CREB in the absence of forskolin had a minimal (1.5-fold) effect on the activity of the reporter. BRCA1 alone had no effect on the activity of the G5-Luc reporter. Transfection of BRCA1 in combination with Gal4-ATF1 in the presence of forskolin stimulated the CRE-Luc reporter gene 3-fold (Fig. 4). We next tested the ability of mutant BRCA1 species to augment CRE-Luc reporter activity. The tumor-derived mutants C61G and C64G both showed differential activation compared with wild-type BRCA1 when expressed in the context of the full-length protein. BRCA1 C61G showed an ~40% reduction in its ability to enhance transactivation from the CRE-Luc reporter gene.

**BRCA1 Modulates Transactivation through the cAMP Response Element**—To assess whether BRCA1 is involved in the functional activation of CREB/ATF transcription factors, we tested the ability of BRCA1 to stimulate transcription from a CRE reporter gene. The activity of the CRE-Luc reporter plasmid measures activation by CREB/ATF family members endogenously present in 293T cells. Expression of BRCA1 alone, in the absence of exogenous forskolin, did not result in activation of the CRE-Luc reporter gene (Fig. 5B). Expression of BRCA1 in the presence of forskolin stimulated the CRE-Luc reporter gene 3-fold (Fig. 5A). We next tested the ability of mutant BRCA1 species to augment CRE-Luc reporter activity. The tumor-derived mutants C61G and C64G both showed differential activation compared with wild-type BRCA1 when expressed in the context of the full-length protein. BRCA1 C61G showed an ~40% reduction in its ability to enhance transactivation from the CRE-Luc reporter gene.

---

2 Y. Houvras and J. D. Licht, unpublished observations.
**Fig. 2. In vitro association of ATF1 with BRCA1.** A, in vitro transcribed/translated ATF1 was incubated with either of two GST-BRCA1 fusion proteins (N-terminal 101 or 304 amino acids) or GST alone. Radiolabeled bound proteins were visualized by fluorography. Input represents 10% of the in vitro transcribed/translated protein incubated with GST-BRCA1. B, GST-ATF1 was used as a capture reagent with a radiolabeled BRCA1 N-terminal fragment containing residues 1–304. Input represents 1% of the in vitro transcribed/translated protein incubated with GST-ATF1. C, full-length ATF1-(1–271) or ATF1 deleted of the C-terminal DNA-binding domain (ATF1-(1–215)) or the N-terminal activation domain (ATF1-(73–271)) was translated in vitro and allowed to interact with GST or GST-BRCA1-(1–301). A schematic diagram of the structure of the ATF1 protein is presented.
DNA-binding domain of ATF1 was required for in vitro interaction with BRCA1 and not the N-terminal KIX domain, which is phosphorylated by protein kinase A and mediates interaction of CREB/ATF family members with CBP. We also found no difference in the interaction between endogenous BRCA1 and ATF1 in the presence or absence of forskolin in MCF-7 cells. These data suggest a model in which BRCA1 binds to the C terminus of ATF1, but cannot modulate the transcriptional activity of the protein unless ATF1 engages the CBP/p300 coactivators through its N-terminal domain.

BRCA1 stimulated the expression of a natural promoter containing a CRE, the TNF-α promoter. CREB and other ATF family members have been shown to be capable of binding to the TNF-α CRE (51). Expression of BRCA1 stimulated the wild-type TNF-α promoter, but failed to stimulate the mutant CRE promoter. These data suggest that BRCA1 can function as a transcriptional coactivator for CREB/ATF family members. Regulation of the TNF-α promoter is controlled by AP1, EGR1, CAAT/enhancer-binding protein-β, and CREB/ATF transcription factors (35, 52–54). Mutation of the TNF-α CRE resulted in a >10-fold decrease in promoter activity, but some residual activity was still present. Intriguingly, TNF-α expression has been linked to hypoxia and both ionizing and UV irradiation in mammalian cells (51, 55, 56). Thus, it is possible that BRCA1 cooperates with CREB/ATF family members to regulate TNF-α in response to DNA-damaging agents.

Several CREB/ATF target genes are directly connected with repair of damaged DNA. In HeLa cells treated with the DNA-
alkylating agent N-methyl-N-nitro-N-nitrosoguanidine, DNA polymerase β, the polymerase involved in nucleotide base excision repair, is up-regulated (57). The transcriptional induction of polymerase β is mediated through a CRE, and treatment with N-methyl-N-nitro-N-nitrosoguanidine triggers CREB phosphorylation. Similarly, UV-C exposure triggers the transcriptional activation of c-Fos through a CRE in its promoter (58). CREB and ATF1 undergo rapid phosphorylation in response to UV-C (59) and may direct the transcriptional activation of target genes involved in DNA damage response. BRCA1 may modulate and augment this activity.

Two viewpoints regarding the function of BRCA1 have emerged in recent years. Substantial evidence implicates BRCA1 in transcription, including association with RNA polymerase holoenzyme (10, 60). On the other hand, BRCA1 association with human RAD51 and BRCA2 and the identification of BRCA1 as a component of a supercomplex of proteins involved in sensing DNA damage and transcription-coupled repair suggest that BRCA1 is critical in the maintenance of genome integrity (reviewed in Ref. 61). These two models of BRCA1 function are not mutually exclusive. Both BRCA1 and ATF1 are phosphorylated in response to DNA damage; and in the case of ATF1, phosphorylation activates docking with co-activators and transcriptional activation. BRCA1 itself may be under the transcriptional control of CREB/ATF family members since a recent report showed that a CRE in the BRCA1 proximal promoter is methylated in breast cancer, correlating with decreased BRCA1 expression (62). The interaction between BRCA1 and ATF1 represents a connection between transcriptional activation and DNA damage response. A critical next step will be to identify genes targeted by ATF1 and BRCA1 in response to DNA damage. Intriguingly, proliferating cell nuclear antigen, a gene whose promoter contains a CRE, is regulated by BRCA1 in response to DNA damage. Intriguingly, proliferating cell nuclear antigen, a gene whose promoter contains a CRE, is regulated by BRCA1 in response to DNA damage. Intriguingly, proliferating cell nuclear antigen, a gene whose promoter contains a CRE, is regulated by BRCA1 in response to DNA damage. Intriguingly, proliferating cell nuclear antigen, a gene whose promoter contains a CRE, is regulated by BRCA1 in response to DNA damage.
51. Taylor, C. T., Fueki, N., Agah, A., Hershberg, R. M., and Colgan, S. P. (1999) J. Biol. Chem. 274, 19447–19454
52. Newell, C. L., Deisseroth, A. B., and Lopez-Berestein, G. (1994) J. Leukocyte Biol. 56, 27–35
53. Tsai, E. Y., Jain, J., Pesavento, P. A., Rao, A., and Goldfeld, A. E. (1996) Mol. Cell. Biol. 16, 459–467, and references therein
54. Yao, J., Mackman, N., Edgington, T. S., and Fan, S. T. (1997) J. Biol. Chem. 272, 17795–17801
55. Hallahan, D. E., Spriggs, D. R., Beckett, M. A., Kufe, D. W., and Weichselbaum, R. R. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 10104–10107
56. Kibitel, J., Hejmadi, V., Alas, L., O’Connor, A., Sutherland, B. M., and Yarosh, D. (1998) Photochem. Photobiol. 67, 541–546
57. Narayan, S., He, F., and Wilson, S. H. (1996) J. Biol. Chem. 271, 18508–18513
58. Englander, E. W., and Wilson, S. H. (1992) DNA Cell Biol. 11, 61–69
59. Iordanov, M., Bender, K., Ade, T., Schmid, W., Sachsenmaier, C., Engel, K., Gaestel, M., Rahmsdorf, H. J., and Herrlich, P. (1997) EMBO J. 16, 1069–1072
60. Scully, R., Anderson, S. F., Chao, D. M., Wei, W., Ye, L., Young, R. A., Livingston, D. M., and Parvin, J. D. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 5605–5610
61. Zhang, H., Tombline, G., and Weber, B. L. (1998) Cell 92, 433–436
62. Mancini, D. N., Rodenhiser, D. I., Ainsworth, P. J., O’Malley, F. P., Singh, S. M., Xing, W., and Archer, T. K. (1998) Oncogene 16, 1161–1169