Critical Residues for Transcription Activation

ABSTRACT
The breast and ovarian cancer susceptibility gene product BRCA1 is a tumor suppressor, but its precise biochemical function remains unknown. The BRCA1 COOH terminus acts as a transcription activation domain, and germ-line cancer-predisposing mutations in this region abolish transcription activation, whereas benign polymorphisms do not. These results raise the possibility that loss of transcription activation by BRCA1 is crucial for oncogenesis. Therefore, identification of residues involved in transcription activation by BRCA1 will help understand why particular germ-line missense mutations are deleterious and may provide more reliable presymptomatic risk assessment.

The BRCA1 COOH terminus (amino acids 1560–1863) consists of two BRCTs preceded by a region likely to be nonglobular. We combined site-directed and random mutagenesis, followed by a functional transcription activation assay in yeast, (a) error-prone PCR-induced random mutagenesis generated eight unique missense mutations causing loss of function, six of which targeted hydrophobic residues conserved in canine, mouse, rat, and human BRCA1; (b) random insertion of a variable pentapeptide cassette generated 21 insertion mutants. All pentapeptide insertions NH2-terminal to the BRCTs retained wild-type activity, whereas insertions in the BRCTs were, with few exceptions, deleterious; and (c) site-directed mutagenesis was used to characterize five known germ-line mutations and to perform deletion analysis of the COOH terminus. Deletion analysis revealed that the integrity of the most COOH-terminal hydrophilic cluster (I1855, L1854, and Y1853) is necessary for activity. We conclude that the integrity of the BRCT domains is crucial for transcription activation and that hydrophobic residues may be important for BRCT function. Therefore, the yeast-based assay for transcription activation can be used successfully to provide tools for structure-function analysis of BRCA1 and may form the basis of a BRCA1 functional assay.

INTRODUCTION
Individuals carrying mutations in the BRCA1 gene have an increased risk of developing breast and ovarian cancer (1). Mutations in BRCA1 alone account for ~45% of families with high incidence of breast cancer and up to 80% of families with both breast and ovarian cancer (2). After an extensive search, BRCA1 was mapped to the long arm of chromosome 17 by linkage analysis (3) and was cloned by positional cloning techniques (4). Human BRCA1 codes for a 1863-amino acid protein with no detectable similarity to known proteins, with the exception of a zinc-binding RING finger domain located in the NH2-terminal region (4), and two BRCT domains found in a variety of proteins involved in cell cycle control and DNA repair (5–7). Recent evidence points to the involvement of BRCA1 in two basic cellular processes: DNA repair and transcriptional regulation. BRCA1 is present in a complex containing Rad51 (8) and BRCA2 (9), and DNA damage may control BRCA1 phosphorylation and subnuclear location (10, 11), strongly suggesting its involvement in the maintenance of genome integrity. Additional evidence for the role of BRCA1 in maintenance of genome integrity is provided by targeted disruption of Brca1 in the mouse. Mouse embryos lacking Brca1 are hypersensitive to γ-irradiation, and cells display numerical and structural chromosomal aberrations (12).

We and others have shown that the BRCA1 COOH terminus has the ability to activate transcription in mammalian and yeast cells and that the introduction of germ-line disease-associated mutations, but not benign polymorphisms, abolishes this activity (13–15). BRCA1 can be copurified with the RNA polymerase II holoenzyme, supporting the idea that BRCA1 is involved in transcription regulation (16, 17). In addition, BRCA1 causes cell cycle arrest via transactivation of p21WAF1/CIP1 (18) and regulates p53-dependent gene expression, acting as a coactivator for p53 (19, 20). In all of these studies, the COOH-terminal region was necessary for activity. It is still not clear whether BRCA1 is a multifunctional protein with repair and transcription regulation functions or whether the role of BRCA1 in repair is mediated through transcription activation. In either case, these functions are not necessarily mutually exclusive.

The dearth of knowledge concerning the precise biochemical function of BRCA1 is a major hurdle in developing a functional test to provide reliable presymptomatic assessment of risk for breast and ovarian cancer. The available data derived from linkage analysis indicate that all mutations that cause premature termination (even relatively subtle mutations such as the deletion of 11 amino acids from the COOH terminus) will confer high risk (21). However, a considerable number of mutations result in amino acid substitutions that, in the absence of extensive population-based studies or a functional assay, do not allow assessment of risk. Two related yeast-based assays designed to characterize mutations in the BRCA1 COOH terminal region have generated results that provide an excellent correlation with genetic linkage analysis (13, 14, 22). This led us to propose the general use of a yeast-based assay to provide functional information and a more reliable risk assessment (23).

In this report, we use site-directed and random mutagenesis to generate mutations in the BRCA1 COOH terminal region that disrupt transcription activation with the intention of both defining critical residues for BRCA1 function and deriving general rules to predict the impact of a particular mutation.

MATERIALS AND METHODS

EAST-BASED FUNCTIONAL ASSAY FOR BRCA1

Yeast Transformation. The GAL4 DBD fusion of the wild-type human BRCA1 COOH terminal region (amino acids 1560–1863) was described previously (13). Alternatively, this fragment was subcloned into the yeast expression vector pLex9 (25) in-frame with the DBD of LexA. Both plasmids have TRP1 as a selectable marker, allowing growth in the absence of tryptophan. We noticed that our previously described BRCA1 (amino acids 1560–1863) construct (13) was made with a 3′ primer lacking a termination codon. This introduces 16 exogenous amino acids to the COOH-terminal region of BRCA1. We have corrected this by using primer 24ENDT (5′-GGCGATCTTCGTGGCTGGGGAAGG-3′). We compared both constructs and ascertained that qualitatively and quantitatively, they have the same activity (not shown).

BRCA1 deletion mutants were generated by PCR on a BRCA1 (amino acids 1560–1863) context using pCR-BRCA1–385 (a gift from Michael Erdos, National Human Genome Research Institute) as a template and the following primers: H1860X (S9503101, 5′-CCGAATTCGAGGGAACCCCTTACGTG-3′); S970074, 5′-GGGATTCTCAGGGATGTTGTCC-3′; and EGY48 [92]. Yeast transformants will produce β-galactosidase, and H74 transformants will grow in medium lacking histidine.

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containing plates. Plasmid DNA was isolated from white colonies (which contain only pLex9::BRCA1 COOH-terminal::Tn4430), and the insertion of Tn4430 into the BRCA1 COOH terminal region was confirmed by restriction enzyme mapping. For the identification of Tn4430 insertions by physical means, pooled plasmid DNA from E. coli consisting of the target plasmid into which Tn4430 was inserted was digested with EcoRI and BamHI, enzymes which liberate the BRCA1 insert but do not cut Tn4430. This digestion of pooled plasmid DNA generates four fragments: the pLex9 vector backbone, the pLex9 vector containing Tn430 insertions, the BRCA1 COOH-terminal fragment, and the BRCA1 COOH terminal fragment containing Tn4430 insertions. The latter fragment was recovered from an agarose gel and recloned in EcoRI-BamHI-digested pLex9 to produce a library of pLex9::BRCA1 COOH terminal domain plasmids containing Tn4430 insertions in the BRCA1 COOH terminal region. In the case of Tn4430 insertions identified by either genetic or physical means, following further restriction mapping the bulk of Tn4430 was deleted from selected clones by digestion with KpnI and religation. The positions of the 15-bp insertions were determined by sequence analysis. Twenty-one plasmids harboring the BRCA1 COOH terminal region with 15-bp insertions were analyzed for transcription activation in S. cerevisiae EGY48 containing pSH18–34.

Western Blots. Yeast cells were grown in selective media to saturation, and A600 was measured. Cells were harvested and lysed in cracking buffer (8 m urea, 5% SDS, 40 mM Tris-HCl pH 6.8, 0.1 mM EDTA, and 0.4 mg/ml bromphenol blue; used 100 µl/7.5 total A600 containing protease inhibitors. The samples were boiled and separated on a 10% SDS-PAGE. The gel was electrobotted onto a wet apparatus to a polyvinylidene difluoride membrane. The blots were blocked overnight with 5% skim milk using TBS-Tween and incubated with the α-pLexA (for LexA constructs) or α-GAL4 DBD (for GAL4 constructs) monoclonal antibodies (Clontech) using 0.5% BSA in TBS-Tween. After four washes, the blot was incubated with the α-mouse IgG horseradish peroxidase conjugate in 1% skim milk in TBS-Tween. The blots were developed using an enhanced chemiluminescent reagent (DuPont NEN, Boston, MA).

RESULTS

Germ-Line Mutations. We analyzed missense mutations occurring in the region from amino acid 1560 to amino acid 1863 described in the Breast Cancer Information Core5 database. To date, 63 missense variants representing mutations in 55 different residues have been documented, most of which have not been characterized either as disease-associated or as benign polymorphisms. Only four missense mutations have been either confirmed or considered very likely to be associated with disease: A1708E (31–33), P1749R (34), R1751Q (33), and M1775R (4, 31, 35). Three of these four mutations target hydrophobic residues that are conserved in canine, mouse, and rat Brca1. (Table 1). Interestingly, the screen revealed that hydrophobic residues were the major targets of mutation (six of eight). Furthermore, all of the targeted residues are perfectly conserved in canine, mouse, and rat Brca1 (Table 1). Even conservative mutations may not be well accepted in residues that are perfectly conserved in all species. This is illustrated by mutation F1761I, where a smaller hydrophobic residue is not tolerated in place of a bulkier one. Loss-of-function mutations were located primarily in the BRCT domains. In particular, mutations that occur in BRCT-C [the most COOH-terminal BRCT (amino acids 1756–1855); BRCT-N (amino acids 1649–1736)] is in residues that constitute the hydrophobic region of the mutants were expressed at levels comparable with the wild type, ruling out the possibility that loss of function was attributable to instability of the protein (Fig. 1). It is important to stress, however, that protein levels are relatively variable in different yeast clones carrying the same constructs and should only be taken as a rough estimate.

Pentapeptide Scanning Mutagenesis Reveals Buried Regions Necessary for Activity. The BRCA1 COOH terminal region was subjected to pentapeptide scanning mutagenesis in which a variable, 5-amino acid cassette was introduced at random. The resulting set of mutated proteins included mutants that displayed complete loss of activity, mutants with reduced activity, and mutants with similar or

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Table 1 Missense mutations leading to loss of function (PCR-mediated mutagenesis screen)

<table>
<thead>
<tr>
<th>Exon</th>
<th>Mutation</th>
<th>Dog*</th>
<th>Mouse*</th>
<th>Rat*</th>
<th>Nucleotide*</th>
<th>Base change</th>
<th>Comments and probable secondary structure element*</th>
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<td>M1652K</td>
<td>M</td>
<td>M</td>
<td>M</td>
<td>5074</td>
<td>T to A</td>
<td>Residue mutated in germ line (M1652T, M1652L).</td>
</tr>
<tr>
<td>18</td>
<td>K1702E</td>
<td>K</td>
<td>K</td>
<td>K</td>
<td>5233</td>
<td>A to G</td>
<td>α-Helix 2 of BRCT-N.</td>
</tr>
<tr>
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<td>Y1703H</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>5226</td>
<td>T to C</td>
<td>α-Helix 2 of BRCT-N.</td>
</tr>
<tr>
<td>18</td>
<td>L1703P</td>
<td>L</td>
<td>L</td>
<td>L</td>
<td>5233</td>
<td>T to C</td>
<td>Found in two independent clones. Located just after α-helix 2 of BRCT-N.</td>
</tr>
<tr>
<td>21</td>
<td>F1761S</td>
<td>F</td>
<td>F</td>
<td>F</td>
<td>5401</td>
<td>T to C</td>
<td>BRCT-N/BRCT-C interval.</td>
</tr>
<tr>
<td>21</td>
<td>F1761I</td>
<td>F</td>
<td>F</td>
<td>F</td>
<td>5400</td>
<td>T to A</td>
<td>α-Helix 1 of BRCT-C. Hydrophobic residue conserved in BRCT superfamily. Mediates interaction between α-helix 1 and α-helix 3.</td>
</tr>
<tr>
<td>22</td>
<td>L1780P</td>
<td>L</td>
<td>L</td>
<td>L</td>
<td>5458</td>
<td>T to C</td>
<td>β-Strand 4 of BRCT-C. Residue mutated in germ line (V1833M) and found in two independent clones. Hydrophobic residue conserved in BRCT superfamily.</td>
</tr>
<tr>
<td>24</td>
<td>V1833E</td>
<td>V</td>
<td>V</td>
<td>V</td>
<td>5617</td>
<td>T to A</td>
<td></td>
</tr>
</tbody>
</table>

* Amino acid corresponds to the predicted translation from canine Brcal cDNA deposited in GenBank accession no. U50709.
* Amino acid corresponds to the predicted translation from murine Brcal cDNA deposited in GenBank accession no. U68174.
* Amino acid corresponds to the predicted translation from rat Brcal cDNA deposited in GenBank accession no. A036760.
* Nucleotide numbering corresponds to human Brcal cDNA deposited in GenBank accession no. U14680.
higher activity than wild type. Table 2 groups the insertions by location: the first group includes mutations in the region NH2-terminal to the BRCT domains (amino acids 1560–1649); the second group contains mutations in BRCT-N; and the third group includes mutations in the intervening region between BRCT-N and BRCT-C. The last group includes mutations in BRCT-C. None of the insertions NH2-terminal to the BRCT domains had a negative effect on transcriptional activation. Also, insertions in the interval between the BRCT domains or at its boundary (1723RGTPI) had generally less drastic effects. In contrast, all insertions within BRCT-N and several within BRCT-C had a more severe effect. It is clear that BRCT-C tolerates insertions better (only three of five showed loss of activity) than BRCT-N (all mutations reduced activity with six of seven showing drastic impairment). The difficulty in predicting the outcome of mutations can be well exemplified by mutations 1824GGTPI and 1822GVPLH. Both of these mutations target residues at the end of BRCT-C α-helix 2, do not change the net charge of the protein, and are only two residues apart. However, 1822GVPLH has ~6% of the wild-type activity, whereas 1824GGTPI has an activity ~80% higher than wild type. Interestingly, the 1793GVPLK insertion increased transcriptional activation ~4-fold, suggesting that this region of BRCA1 may directly contact a component of the transcription machinery. The pentapeptide mutagenesis results demonstrated that, in addition to substitution mutations, insertion mutagenesis in the COOH-terminal region, particularly in the BRCT domains, can profoundly alter transcriptional activity by BRCA1.

**Characterization of Germ-Line Mutations.** To assess the activity of variants that have already been documented but not characterized, we decided to introduce a set of mutations and assay for transcription activation in yeast (Table 3). Mutations T1561I and L1564P are both located in the region preceding the BRCT domains and displayed wild-type activity. L1564P was expected to be a polymorphism because proline is the residue found in the rat Brca1 sequence. The three remaining variants are localized to the BRCT domains. Two variants, D1733G and P1806A, displayed wild-type activity and are suggested to be benign polymorphisms. D1733G introduces a glycine that probably does not affect BRCT structure. P1806A involves a conservative change, and it is important to note that the rat Brca1 sequence has leucine in that position. Only one of the variants tested, G1738E, displayed a loss of function phenotype. Thus, we propose that G1738E is a disease-predisposing variant.

**Deletion Mutants of COOH-Terminal Residues Define the Minimal Transactivation Domain (MTD).** A construct carrying the germ-line mutation Y1853X does not have detectable transcriptional activity in the context of a GAL4 DBD fusion of the BRCA1 COOH terminus (amino acids 1560–1863; Refs. 13 and 15). A construct containing amino acids 1760–1863 can be considered the MTD, defining I1760 as a 5’ border of this domain (13, 15). Thus, the NH2-terminal border of the MTD coincides closely with the NH2-
terminal border of BRCT-C (I1760 is the first conserved hydrophobic residue in the BRCT superfamily). To identify the COOH-terminal border of the MTD, several deletion mutants were made in the amino acids 1560–1863 context and assayed for their ability to activate transcription in yeast. Fig. 2 shows the several deletion mutants analyzed aligned to mouse, rat, dog, and human BRCA1 wild-type sequences. Mutant H1860X introduces a stop codon but maintains all of the conserved amino acids in canine and human BRCA1. P1856X maintains the hydrophobic residues, which are conserved in all of the BRCT domains described in several species. I1855X and L1854X delete one and two conserved hydrophobic residues, respectively. Y1853X is a mutation found in the germ-line of breast and ovarian cancer patients in high-risk families (21). These constructs were transformed into SFY526 and HF7c and analyzed for their ability to activate different reporters (Fig. 2b). Activity comparable with the wild-type was obtained with mutants H1860X and P1856X. However, mutations that disrupted the conserved hydrophobic residues (I1855X and L1854X) at the end of the BRCT domain abolished activity. Therefore, we define the MTD in BRCA1 as amino acids 1760–1855. To determine whether the loss of activity by the mutants correlated with the stability of the protein, yeast cells were transformed with the same mutated alleles in a vector conferring high expression (pAS2-1). Transcriptional activity using these constructs (in pAS2-1 backbone) was measured, and results were similar with I1855X showing some residual activity. Expression was highly variable, and mutants were in general expressed at lower levels than wild type (Fig. 2c). There was no correlation between loss of activity and lower levels of expression because the transcriptionally active mutant H1860X was expressed at levels lower or comparable with transcriptionally inactive mutants I1855X and Y1853X (Fig. 2c).

**DISCUSSION**

In this report, we describe an extensive mutagenesis analysis of the BRCA1 COOH terminal region and partly define the critical requirements for transcriptional activity by BRCA1. Four complementary strategies were used: (a) error-prone PCR mutagenesis, followed by a screen for loss of function; (b) pentapeptide scanning mutagenesis; (c) site-directed mutagenesis to analyze documented mutations; and (d) deletion analysis of the COOH terminus. Our results support the notion that there are no particular hot spots for loss-of-function mutations, but rather that these mutations are scattered throughout the coding sequence. Nevertheless, we were able to identify preferential sites critical for activation. An overview of the mutations and their effects is presented in Fig. 3. We discuss the general conclusion of each strategy and then we analyze the possible structural outcome of the mutations based on the crystal structure of XRCC1 BRCT (36).

**Error-prone PCR Mutagenesis.** Eight distinct BRCA1 mutations were recovered that resulted in loss of transcription activation function. In the course of the screening procedure, many additional clones that displayed a light blue color were noted and were probably mutants with reduced function, but only clones with complete loss of function were analyzed further. No PCR-generated mutations were found in the region external to the BRCT domains, although this constitutes approximately one-third of the tested sequence, indicating a preference for mutations that affect transcription activation to occur in the BRCT domains (Fig. 3).

Six of eight unique PCR-generated mutations were in hydrophobic residues conserved in human, canine, mouse, and rat Brca1 (6, 7), supporting the notion that hydrophobic residues are important for the stability of the BRCT domains and BRCA1 function in vivo.

**Table 3 Transcriptional activity of human BRCA1 unclassified variants (amino acids 1560–1863)**

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<th>Exon</th>
<th>Mutation</th>
<th>Activity</th>
<th>Dog</th>
<th>Mouse</th>
<th>Rat</th>
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<td>16</td>
<td>T1561I</td>
<td>+</td>
<td>A</td>
<td>T</td>
<td>T</td>
<td>4801</td>
<td>C to T</td>
<td>Unknown</td>
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<td>L1564P</td>
<td>+</td>
<td>L</td>
<td>P</td>
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<td>BRCT-N/BRCT-C interval</td>
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<td>β-Strand 2/β-strand 3 loop of BRCT-C</td>
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* At least 10 independent clones were assayed and scored 8 h after the addition of X-gal. +, blue with same intensity as wild-type control; −, white, similar to two (F1761S and Y1769X) loss-of-function controls.

### YEAST-BASED FUNCTIONAL ASSAY FOR BRCA1

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**Fig. 2. Deletion analysis of the COOH-terminal region.** a, alignment of the wild-type sequences of the COOH terminus of rat, mouse, dog, and human BRCA1. Amino acids in bold represent conserved residues. Shaded area, residues at the 3′ border of the BRCT-C domain. b, transcriptional activity of GAL4 DBD fusion deletion constructs, made in the context of BRCA1 amino acids 1560–1863. S. cerevisiae (H77c) carrying the indicated fusion proteins were assayed for growth in the absence of tryptophan and histidine in liquid medium. Activity relative to cells growing in medium lacking tryptophan alone after 36 h is shown in parentheses. Filter β-galactosidase assays for SFY526 were scored at 12 h after X-gal addition. At least four independent clones were assayed for each construct. c, Western blot showing levels of protein expression of the different constructs (black arrow) detected by a α-GAL4-DBD monoclonal antibody.

**Reference**

- Zhang et al. (36)
- Murine Brca1 CDNA deposited in GenBank accession no. U50709.
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- According to a BRCA1 BRCT model from Zhang et al. (36).

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</table>

* At least 10 independent clones were assayed and scored 8 h after the addition of X-gal. +, blue with same intensity as wild-type control; −, white, similar to two (F1761S and Y1769X) loss-of-function controls.

### YEAST-BASED FUNCTIONAL ASSAY FOR BRCA1

Error-prone PCR mutagenesis. Eight distinct BRCA1 mutations were recovered that resulted in loss of transcription activation function. In the course of the screening procedure, many additional clones that displayed a light blue color were noted and were probably mutants with reduced function, but only clones with complete loss of function were analyzed further. No PCR-generated mutations were found in the region external to the BRCT domains, although this constitutes approximately one-third of the tested sequence, indicating a preference for mutations that affect transcription activation to occur in the BRCT domains (Fig. 3).

Six of eight unique PCR-generated mutations were in hydrophobic residues conserved in human, canine, mouse, and rat Brca1 (6, 7), supporting the notion that hydrophobic residues are important for the stability of the BRCT domains and BRCA1 function in vivo.

**Fig. 2. Deletion analysis of the COOH-terminal region.** a, alignment of the wild-type sequences of the COOH terminus of rat, mouse, dog, and human BRCA1. Amino acids in bold represent conserved residues. Shaded area, residues at the 3′ border of the BRCT-C domain. b, transcriptional activity of GAL4 DBD fusion deletion constructs, made in the context of BRCA1 amino acids 1560–1863. S. cerevisiae (H77c) carrying the indicated fusion proteins were assayed for growth in the absence of tryptophan and histidine in liquid medium. Activity relative to cells growing in medium lacking tryptophan alone after 36 h is shown in parentheses. Filter β-galactosidase assays for SFY526 were scored at 12 h after X-gal addition. At least four independent clones were assayed for each construct. c, Western blot showing levels of protein expression of the different constructs (black arrow) detected by a α-GAL4-DBD monoclonal antibody.

**Reference**

- Zhang et al. (36)
- Murine Brca1 CDNA deposited in GenBank accession no. U50709.
- Amino acid corresponds to the predicted translation from murine Brca1 CDNA deposited in GenBank accession no. U68174.
- Amino acid corresponds to the predicted translation from canine Brca1 CDNA deposited in GenBank accession no. AF036760.
Pentapeptide Scanning Mutagenesis. Pentapeptide scanning mutagenesis is a method by which a variable 5-amino acid cassette is introduced at random into a target protein (29, 30, 37). This approach differs from error-prone mutagenesis because clones are not selected for loss of activity but rather mutations are analyzed only after they have been generated. Therefore, mutants with gain of function, loss of function, and novel activities can be produced (30, 37). Moreover, it has been shown that insertion is essentially random (29). The results obtained are in agreement with the PCR-mediated mutagenesis in that the region NH2-terminal to the BRCT domains (amino acids 1560 –1649) seems to be more tolerant of mutation; none of six different pentapeptide insertions in this region affected transcription activation. The fact that derivatives containing insertion mutations in this region retained wild-type activity suggests that this region is nonglobular and is probably a flexible part of the COOH-terminal region without many critical secondary structure elements. In fact, the region encompassing amino acids 1524 –1661 is predicted to be nonglobular (5). The pentapeptide mutagenesis results also suggest that changing the net charge of the protein does not necessarily correlate with an alteration in transcription activity, as would be expected for classical acidic activators (38), because 1793GVPLK (which adds a positive charge) shows a 4-fold increase in activity. Interestingly, only 4 of the 63 COOH-terminal germ-line variants involve nonconservative substitutions in acidic residues, thought to be important for activation, suggesting that, contrary to initial predictions, BRCA1 may not be a classical acidic activator (4). The 1793GVPLK mutation, which is hyperactive for transcription activation, may define a point of contact between the BRCA1 COOH-terminal region and the transcription machinery.

Deletion Analysis. Our analysis demonstrates that residues COOH-terminal to amino acids 1855 are dispensable for activation, consistent with the extreme evolutionary divergence of those residues (Fig. 2; Refs. 39 and 40). The results also underscore the importance of the last hydrophobic cluster in the sequence (YLI for human and canine; YLV for mouse and rat) and provide a plausible explanation for the complete loss of function (in vitro and in vivo) of Y1853X alleles.

Site-directed Mutagenesis. Only one of five germ-line mutations analyzed displayed loss of function, suggesting that a large part of variants in the COOH-terminal region will probably be benign polymorphisms, including some variants found in the BRCT domains. Very little data are available at this moment to confirm or contradict the results obtained. In particular, T1561I illustrates the difficulties involved in predicting outcome from population data. T1561I was found in one affected individual but not in control individuals (41). This could suggest that T1561I is a disease-predisposing variant. However, although found as a germ-line mutation, it was absent from

Fig. 3. Domain structure of the BRCA1 COOH terminal region (amino acids 1560 –1863) and characterized mutations. Top panel, a schematic representation of full-length BRCA1 protein featuring the RING domain (yellow box) in the NH2-terminal region and the BRCT domains (red circles) in the COOH-terminal region. The region analyzed in this study is contained in the red box, which is enlarged and represented in the bottom panel. Purple and pink bars, predicted β-strands and α-helices, respectively. Secondary structure predictions were made by Zhang et al. (36) based on the crystal structure of the XRCC1 BRCT domain. Mutations represented in the upper part (red triangles) result in loss of function, whereas mutations in the lower part (green triangles) result in activity equal or higher than wild type. Germ-line mutations and polymorphisms are variants defined by genetic linkage to be disease-associated and benign polymorphisms, respectively. Site-directed mutagenesis, PCR mutagenesis, transposon-mediated mutagenesis, and deletion analysis represent mutations that have been characterized by transcription activation assay in yeast to be either loss of function (upper part) or wild type (lower part).
the tumor from the same patient (41), indicating that this mutation is a benign polymorphism.

**Structural Basis for Effects of BRCT Domain Mutations.** The COOH-terminal BRCT domain of XRCC1 consists of a four-stranded parallel β-sheet (β1–β4) surrounded by three α-helices (α1–α3; Ref. 36). The β-sheet forms the core of the structure with a pair of α-helices (α1 and α3) on one side of the β-sheet and the remaining α-helix (α2) on the other side. A model of the more COOH-terminal BRCT domain of BRCA1 has been constructed based on the crystal structure of the BRCT domain of XRCC1 (36). This model allows an interpretation of the effect of some of the mutations described in this study (Tables 1–3) on BRCT domain structure (Fig. 3).

The position of the M1652K mutation corresponds to a position (Asp4) in the XRCC1 structure that is thought to form a salt bridge at the BRCT dimer interface (36). Although M1652 would not be expected to be involved in salt bridge formation at neutral pH, residues in this region nevertheless may also be involved in homo- or heterodimer formation in BRCA1.

Missense mutations at positions 1702, 1703, and 1705 of the BRCT-N domain and a pentapeptide insertion at position 1822 of the BRCT-C domain abolish transcription activation by the BRCA1 COOH terminus (Tables 1 and 2). These mutations are predicted to occur in a region of highly variable length and composition that encompasses helix α2 in BRCT domains (36). It was suggested that this variability indicated that this region was not involved in formation of the core fold of the BRCT domain (36). Nevertheless, the mutations isolated here reveal that this region of the BRCT domain is critical for the transcription activation function of the BRCA1 COOH terminus.

Residue F6 forms part of a highly conserved hydrophobic pocket centered on residue W74 in helix α3 in the COOH-terminal BRCT domain of XRCC1 (36). Mutations at the corresponding position (F1761) in the BRCT-C domain of BRCA1 abolish transcription activation (Table 1). By analogy with XRCC1, residue F1761 of BRCA1 is also predicted to form part of a hydrophobic pocket, the disruption of which by mutation may compromise correct BRCT domain folding. In contrast, residue L25 is implicated in the interactions between helices α1 and α3, which form a paired helical bundle in the three-dimensional structure of the BRCT domain of XRCC1 (36). A missense mutation of the corresponding residue (L1780) or a pentapeptide insertion at this position in the BRCT-C domain of BRCA1 abolishes transcription activation by the BRCA1 COOH terminus region (Tables 1 and 2). These mutations are likely to affect the interactions between helices α1 and α3, thereby destabilizing the BRCT domain structure. Two other missense mutations in the BRCT-C domain, P1806A and V1833E, were shown, respectively, to display wild-type activity and to abolish transcription activation (Tables 1 and 3). Interestingly, P1806A is predicted to have no obvious effect on the structure, whereas a less drastic mutation at position V1833 (to methionine) has been predicted to destabilize the fold of the domain (36), suggesting that V1833E will behave similarly.

Pentapeptide insertions in many of the predicted secondary structure elements in the COOH-terminal region of BRCA1 abolish transcription activation (Table 1 and Fig. 3). Some of these insertions are likely to disrupt formation of the correct BRCT domain core fold, e.g., insertions in strand β2 (1676GRTGL) and in helices α2 (1822GVPPLH) and α3 (1717WGTFP). In contrast, the 1780GVQL insertion in helix α1 is predicted to be at the BRCT dimer interface and thereby may affect the association of this domain with another protein, e.g., RNA helicase A, which interacts with BRCA1 through residues in helix α1 (17).

**Different Roles of BRCT-N and BRCT-C.** Our insertion mutagenesis results suggest that BRCT-C can tolerate insertions better than BRCT-N without affecting transcription activation function. In addition, BRCT-N is more highly conserved in other species than is BRCT-C (39, 40), suggesting a higher constraint for function. The BRCT-N seems to be very important for binding to RNA helicase A (17), although it seems to lack an independent activation domain (mutant Y1769X is inactive). The borders of BRCT-C coincide well with the limits of the MTD, but only in combination with BRCT-N are high levels of activation achieved (13). It is tempting to speculate that BRCT-N is involved in the interaction of BRCA1 with RNA helicase A and is responsible for presenting BRCT-C in a correct way to obtain a transcriptionally competent activator.

**Functional Assay.** We have performed an extensive analysis of the BRCA1 COOH terminal region (amino acids 1560–1863) and have found that there is a correlation between loss of transcription activation function and the human genetic data, suggesting that the assay could be used to predict the effect of missense mutations in this region. Although the effects of mutations on transcriptional activity have been found to be comparable in yeast and mammalian cells (13, 15), it is possible that the effect of some mutations may be evident only in mammalian cells, e.g., because of an interaction with mammalian-specific regulators, raising the possibility of a misinterpretation of the data obtained in yeast.

In the results presented here for substitution mutations, we have used a reporter gene with relatively low stringency (eight Lex operators; Ref. 26). The rationale for this choice was to recover only mutants that cause dramatic reduction or complete loss of activity. Mutations that partially disrupt the function would still activate the reporter. In the absence of knowledge of the minimum in vivo threshold of transcriptional activity needed for tumor suppression, it would be inappropriate to make decisions on whether a particular mutation would represent a wild-type or a cancer predisposing allele. For example, a particular mutation that shows 50% loss of activity in yeast could still be perfectly functional in breast and ovarian cells.

In conclusion, the data presented here suggest that the yeast assay for monitoring transcription activation by BRCA1 will provide a wealth of functional information in a research setting. That includes identifying protein-protein interaction regions, defining critical residues for activity, and providing tools to identify possible regulators. A general use of the assay to help in risk assessment and providing information for clinical decisions must await further confirmation from population-based studies.

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