Functional analysis of BRCA1 C-terminal missense mutations identified in breast and ovarian cancer families

Johan Vallon-Christersson1, Charmagne Cayanan7, Karin Haraldsson1, Niklas Loman1, Jon Thor Berghorsson3, Karen Brøndum-Nielsen4, Anne-Marie Gerdes5, Pål Møller6, Ulf Kristoffersson2, Håkan Olsson1, Åke Borg1 and Alvaro N.A. Monteiro7,8,+

1Department of Oncology and 2Department of Clinical Genetics, University Hospital, SE-221 85 Lund, Sweden, 3Department of Pathology, University Hospital of Iceland, IS-121 Reykjavik, Iceland, 4The John F. Kennedy Institute, 2600 Glostrup, Denmark, 5Department of Clinical Genetics, University Hospital, 2100 Copenhagen, Denmark, 6Unit of Medical Genetics, the Norwegian Radium Hospital, N-0310 Oslo, Norway, 7Laboratory of Molecular Oncology, Strang Cancer Prevention Center, New York, NY 10021, USA and 8Department of Cell Biology, Weill Medical College of Cornell University, New York, NY 10021, USA

Received 5 September 2000; Revised and Accepted 12 December 2000

Germline mutations in the breast and ovarian cancer susceptibility gene BRCA1 are responsible for the majority of cases involving hereditary breast and ovarian cancer. Whereas all truncating mutations are considered as functionally deleterious, most of the missense variants identified to date cannot be readily distinguished as either disease-associated mutations or benign polymorphisms. The C-terminal domain of BRCA1 displays an intrinsic transactivation activity, and mutations linked to disease predisposition have been shown to confer loss of such activity in yeast and mammalian cells. In an attempt to clarify the functional importance of the BRCA1 C-terminus as a transcription activator in cancer predisposition, we have characterized the effect of C-terminal germline variants identified in Scandinavian breast and ovarian cancer families. Missense variants A1669S, C1697R, R1699W, R1699Q, A1708E, S1715R and G1738E and a truncating mutation, W1837X, were characterized using yeast- and mammalian-based transcription assays. In addition, four additional missense variants (V1665M, D1692N, S1715N and D1733G) and one in-frame deletion (V1688del) were included in the study. Our findings demonstrate that transactivation activity may reflect a tumor-suppressing function of BRCA1 and may form the basis of a functional assay.

INTRODUCTION

Germline mutations in the breast and ovarian cancer susceptibility gene BRCA1 (OMIM 113705) predispose carriers to early-onset breast and breast–ovarian cancer (1,2) and it is estimated that ~5% of all breast cancer cases are caused by inherited mutations in dominant disease genes. The majority of familial cases with both breast and ovarian cancer and a substantial part of families with breast cancer alone involve mutations in BRCA1 (3). The BRCA1 gene product is an 1863 amino acid phosphoprotein with a RING-finger motif at its N-terminus and two BRCA1 C-terminal (BRCT) domains at its C-terminus (1,4). With the exception of these domains, BRCA1 displays no similarity to other known proteins. The BRCT domains are mainly found in proteins involved in DNA repair, recombination and cell cycle control (5,6). Early findings suggest that BRCA1 is a tumor suppressor because loss of the wild-type allele was observed in familial breast and ovarian cancer cases (7). Although the function of BRCA1 remains unclear, there is increasing support for a role in DNA repair and transcription activation (for reviews see refs 8 and 9). BRCA1 interacts with large protein complexes involved in DNA repair such as Rad51/BRCA2 (10,11) and Rad50/Mre11/p95 (12,13). Importantly, BRCA1 becomes hyperphosphorylated and disperses from Rad51-containing nuclear foci in response to DNA damage (14,15). In mice, Brca1 is required for transcription-coupled repair of oxidative DNA damage (16) and Brca1–/– embryonic cells accumulate genetic aberrations (17). However, no direct mechanism of action has been described which explains how BRCA1 exerts its functions.

*To whom correspondence should be addressed at: Laboratory of Molecular Oncology, Strang Cancer Prevention Center, The Rockefeller University, Box 231, 1230 York Avenue, New York, NY 10021, USA. Tel: +1 212 7340567 (ext. 225); Fax: +1 212 4729471; Email: monteia@rockvax.rockefeller.edu
A transactivation activity was first ascribed to BRCA1 by demonstrating that, when fused to a heterologous DNA-binding domain, the C-terminus of BRCA1 acts as a transcription activator (18,19). BRCA1 associates in vivo with RNA polymerase II (pol II) holoenzyme as well as with the core pol II (20–22) and modulates transcription mediated by several transcription factors (9 and references therein).

The discovery of a transactivation activity revealed a testable function of BRCA1 and yeast-based assays have been proposed as a means of characterizing missense variants because disease-associated mutations abolish this activity (23,24). Numerous mutations in BRCA1 have been described and established as disease-associated (Breast Cancer Information Core database, BIC). Such mutations are located throughout the gene and typically result in premature translation termination. Apart from a handful of clearly linked or strongly suspected disease-associated mutations, most amino acid substitutions reported hitherto cannot readily be distinguished as either disease-associated or benign polymorphisms and are classified as variants of uncertain significance (BIC), posing a very relevant problem in genetic counseling. Nevertheless, although the precise biochemical function of the protein remains unknown, increasing knowledge of the structural properties and biological roles of BRCA1 provides support in discriminating these alterations, eventually allowing functional assays to be developed (24,25). Yeast-based assays have been able to discriminate between disease-associated mutations and benign polymorphisms in the C-terminus of BRCA1 (18,24,26,27). Therefore, it is tempting to suggest that the transactivation activity reflects a tumor-suppressing function of BRCA1 in vivo. Here we use a transcription activation assay to characterize the effect of unique germline variants identified in Scandinavian breast and ovarian cancer families. Seven of the included variants are of missense type and one is of nonsense type. In addition, we analyzed five C-terminal BRCA1 variants reported by others (BIC).

RESULTS

Analysis of hereditary breast and ovarian cancer has revealed several novel as well as previously described variants of BRCA1. Patients have been screened for mutations in BRCA1 and BRCA2 as described by Hakansson et al. (28). Here we analyze missense variants and one truncating mutation that localize to the C-terminal region of BRCA1 (Fig. 1). These variants were not found in 50 healthy Swedish control individuals (no screen has been done for G1738E). Moreover, >450 index cases with familial history of breast–ovarian cancer have been screened for mutations in BRCA1 and the variants reported here have been found only in their respective families, indicating that they represent rare variants.

We introduced the variants in constructs containing the fusion GAL4 DNA-binding domain (DBD):BRCA1 (amino acids 1560–1863) (Fig. 1) (18,26). In order to assess their transactivation activity these constructs were transformed into two Saccharomyces cerevisiae strains, HF7c and SFY526, containing reporter genes under the control of the GAL1 upstream activating sequence (UAS), recognized by GAL4 DBD. Wild-type BRCA1 (amino acids 1560–1863) was used as a positive control and vector without insert was used as a negative control. Results were comparable in both yeast strains in a semi-quantitative assay (Table 1).
### Analysis of variants identified in Lund families

Variants A1669S, R1699W and R1699Q displayed wild-type activity, suggesting that they represent benign polymorphisms (Table 1). For variant A1669S, the data from functional assays are in agreement with the pedigree analysis (Fig. 2). One of the affected family members did not carry the mutation and cases of uterine and very early-onset ovarian cancer indicate involvement of predisposing genes other than *BRCA1* or *BRCA2*. Additional clinical data should provide insight regarding A1669S and will serve as measurement of the prediction provided by the assay. Interestingly, pedigree analysis seemed to indicate that the R1699W is a cancer-predisposing allele (Fig. 2, Lund 279). Disease association is less clear for R1699Q, found in a patient diagnosed with breast cancer at the age of 39 but without familial history of disease. Others found this variant in an unaffected individual, whose mother was diagnosed with premenopausal breast cancer and considered to be an obligate carrier of the R1699Q variant and whose grandmother was diagnosed with ovarian cancer at the age of ~60 years but without known mutation status (T.S. Frank, personal communication). The apparent discrepancy between the family and functional data prompted further examination of the R1699 variants.

### Table 1. Transcriptional activity of *BRCA1* variants identified in Scandinavian breast–ovarian cancer families and variants obtained from the BIC database

<table>
<thead>
<tr>
<th>Family/ source</th>
<th>Exon</th>
<th>Mutation</th>
<th>Dog&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Mouse&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Rat&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Nucleotide&lt;sup&gt;d&lt;/sup&gt;</th>
<th>Base change</th>
<th>Probable secondary structure elements&lt;sup&gt;e&lt;/sup&gt;</th>
<th>Transcriptional activity&lt;sup&gt;f&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lund 275</td>
<td>18</td>
<td>C1697R</td>
<td>C C C</td>
<td>5208</td>
<td>T→C</td>
<td>α-helix 2 of BRCT-N</td>
<td>+</td>
<td>SFY526 (β-gal)</td>
<td></td>
</tr>
<tr>
<td>Lund 279</td>
<td>18</td>
<td>R1699W</td>
<td>R R R</td>
<td>5214</td>
<td>C→T</td>
<td>α-helix 2 of BRCT-N</td>
<td>+</td>
<td>EGY48 (β-gal)</td>
<td></td>
</tr>
<tr>
<td>Lund 488</td>
<td>18</td>
<td>R1699Q</td>
<td>R R R</td>
<td>5215</td>
<td>G→A</td>
<td>α-helix 2 of BRCT-N</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Lund 20</td>
<td>18</td>
<td>A1708E</td>
<td>A A A</td>
<td>5242</td>
<td>C→A</td>
<td>α-helix 2/β-strand 4 loop of BRCT-N</td>
<td>–i</td>
<td>Not done</td>
<td></td>
</tr>
<tr>
<td>Lund 184</td>
<td>18</td>
<td>S1715R</td>
<td>S S S</td>
<td>5262</td>
<td>A→C</td>
<td>β-strand 4/α-helix 3 loop of BRCT-N</td>
<td>–</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>Lund 32</td>
<td>20</td>
<td>G1738E</td>
<td>G G G</td>
<td>5332</td>
<td>G→A</td>
<td>BRCT-N/BRCT-C interval</td>
<td>–</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>Lund 190</td>
<td>24</td>
<td>W1837X</td>
<td>W W W</td>
<td>5630</td>
<td>G→A</td>
<td>α-helix 3 of BRCT-C; conserved W in BRCT domains</td>
<td>–</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>BIC 17</td>
<td>17</td>
<td>V1665M</td>
<td>V V V</td>
<td>5112</td>
<td>G→A</td>
<td>α-helix 1 of BRCT-N</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>BIC 17</td>
<td>17</td>
<td>V1688deI</td>
<td>I I I</td>
<td>5181</td>
<td>delGGT</td>
<td>β-strand 3 of BRCT-N</td>
<td>–</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>BIC 17</td>
<td>17</td>
<td>D1692N</td>
<td>D D D</td>
<td>5193</td>
<td>G→A</td>
<td>β-strand 3/α-helix 2 of BRCT-N</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>BIC 18</td>
<td>18</td>
<td>S1715N</td>
<td>S S S</td>
<td>5263</td>
<td>G→A</td>
<td>β-strand 4/α-helix 3 loop of BRCT-N</td>
<td>–</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>BIC 20</td>
<td>20</td>
<td>D1733G</td>
<td>D E E</td>
<td>5317</td>
<td>A→G</td>
<td>BRCT-N/BRCT-C interval</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>Amino acids correspond to predicted translation from canine *Brca1* cDNA (GenBank accession no. U50709).

<sup>b</sup>Amino acids correspond to predicted translation from murine *Brca1* cDNA (GenBank accession no. U68174).

<sup>c</sup>Amino acids correspond to predicted translation from rat *Brca1* cDNA (GenBank accession no. AF036760).

<sup>d</sup>Nucleotide numbering corresponds to human *BRCA1* cDNA (GenBank accession no. U14680). Alignment was performed using Vector NTI Multiple Sequence Alignment version 1.0.1.1.

<sup>e</sup>According to a *BRCA1* BRCT model from Zhang et al. (29).

<sup>f</sup>Thirty-six individual colonies were streaked on solid SD medium lacking tryptophan and histidine and scored for growth after 2 days at 30°C. A positive score (+) was noted if growth was visually identical to the positive control (wild-type *BRCA1* amino acids 1560–1863) and a negative score (–) was noted if growth was visually identical to the negative control (vector with no insert).

<sup>g</sup>Thirty-six individual colonies were streaked on filter overlaid on solid SD medium and assayed for β-gal activity after 2 days at 30°C. A positive score (+) was noted if the activity was visually identical to the positive control (wild-type *BRCA1* amino acids 1560–1863) and a negative score (–) was noted if the activity was visually identical to the negative control (vector with no insert). Clones were scored 6 h after addition of X-gal.

<sup>h</sup>At least six individual colonies were streaked on filter overlaid on solid SD medium lacking tryptophan and uracil and assayed for β-gal activity the next day at 30°C. A positive score (+) was noted if the activity was visually identical to the positive control (wild-type *BRCA1* amino acids 1560–1863) and a negative score (–) was noted if the activity was visually identical to the negative controls (M1775R and Y1853X). Clones were scored 2 h after addition of X-gal.

<sup>i</sup>Partly reduced β-gal activity.

<sup>j</sup>Published results, Monteiro et al. (18).

<sup>k</sup>Published results, Hayes et al. (24).
mutations (Table 1), an observation in agreement with pedigree analysis for mutations C1697R, A1708E and S1715R (Fig. 2).

The amino acid substitution C1697R is a rather dramatic one, from a non-polar residue capable of forming disulfide linkages to a positively charged residue, located in a critical α-helix based on the structure of XRCC1 BRCT (29). Furthermore, the residue in question is strictly conserved in other BRCA1 homologs (Table 1) (30,31). In addition to family Lund 275, in which it segregates with the disease, the C1697R variant has been found in three other breast cancer patients. One case had multicentric disease at age 35 and a family history of breast cancer (sister and mother), whereas the other two cases had bilateral disease at ages 41 and 44 and mothers with breast/skin cancer and cancer of unknown origin, respectively (J.T. Bergthorsson et al., unpublished data). Thus, combined clinical data indicate association between the variant and breast cancer. Variant A1708E has been reported to the BIC database 14 times, including our finding in Lund 20. It has been previously shown to cause loss of function in different assays (18,19,27) and the presence of A1708E in Lund 20 further demonstrates the variant as a disease-associated mutation. S1715 is an evolutionarily conserved residue. However, the disease pattern in Lund 184 (harboring an S1715R substitution; variant S1715N was also analyzed) is not satisfactorily explained by a mutation in BRCA1 alone because it presents an uncharacteristic phenotype. Multiple cases of colon cancer might suggest the involvement of a mismatch repair gene defect. However, co-segregation between the mutation and breast and ovarian cancer is observed and these cancer forms are predominant among women in the pedigree (Fig. 2). We recently found the G1738E variant, which displayed loss of transactivation activity in our assays, in a young patient affected with bilateral breast cancer and a family history of disease. In addition, others found the variant in a family with a strong pattern of hereditary disease in which the patient carrying the alteration suffered from breast cancer at an early age (T.S. Frank, personal communication). These findings strengthen the correlation between disease predisposition and predictions made by the transcription assay (24).

Analysis of variants in the BIC database

Variants V1665M, D1692N and D1733G displayed wild-type activity, suggesting that they represent benign polymorphisms. Figure 2. Scandinavian breast and breast–ovarian cancer families with germline BRCA1 C-terminal missense or truncating mutations. Cancer types, age at diagnosis and mutation status are shown. *, mutation; °, confirmed from blood sample not to carry mutation; ^, determined from paraffin embedded tumor tissue not to carry mutation. Cancer type: Br, breast; Ov, ovary; Ut, uterus; Gyn, gynecological; Pr, prostate; Co, colon; Rec, rectal; Leuk, leukemia; Lu, lung; Br, brain; Bl, bladder; Pa, pancreas; Ga, gastric; Pen, Penile; Abd, abdominal; Ca, cancer of unknown type; Ca?, possibly affected.
The V1665M variant affects a residue close to A1669, in the predicted BRCT conformation (29), which also displayed wild-type activity (Table 1 and Fig. 3), suggesting that this small stretch is tolerant to mutations. Variant D1692N affects the residue predicted to form a salt bridge with S1715, thereby stabilizing the interactions between BRCT α2 and α4 regions (29). However, D1692N displayed wild-type transactivation activity, suggesting that the predicted salt bridge is not important for the transactivation ability of BRCA1 (Table 1 and Fig. 3). The D1733G variant is a conserved acidic residue located in the BRCT-N/BRCT-C interval. However, more information is still needed for a reliable characterization of this variant.

Variants V1688del and S1715N displayed loss of activity, suggesting that they represent cancer-associated mutations (Table 1). Alteration V1688del is an in-frame deletion of a conserved hydrophobic residue predicted to be part of β3 in the BRCT-N domain (29). Previous mutation analysis has underscored the importance of hydrophobic residues for the function of BRCA1 (24). Similar to S1715R (Lund 184), the substitution S1715N (BIC) resulted in loss of activity in the assay.

Fusion protein and promoter stringency do not influence assay outcome

To rule out the possibility that the results obtained with the GAL4 DBD fusions were dependent on the DBD, we also performed the experiments using fusions to LexA DBD in the S.cerevisiae strain EGY48 (24). A fusion of wild-type BRCA1 (amino acids 1560–1863) was used as a positive control and two mutants defined by genetic linkage as disease-associated, M1775R and Y1853X, were used as negative controls (1,2). Results from the LexA-based and GAL4-based assays were comparable (Table 1).

The reporter genes used in the yeast experiments contain multiple binding sites in their promoters (eight for LexA; four for GAL4), raising the possibility that variants with partial loss of function could score as wild-type in the semi-quantitative filter β-galactosidase assay. This could be particularly important in the case of the R1699W variant for which we found a contradiction between the family data and transcription activity. Therefore, EGY48 experiments with the R1699W variant were performed with the LacZ reporter under the control of one, two or eight LexA operators (32). In all cases, R1699W was indistinguishable from the wild-type allele (data not shown).

Quantitative assessment of transcription activation

Despite the fact that we saw no difference that could be attributed to promoter stringency, it was still possible that variants with partial loss of activity could be differentiated only using quantitative liquid β-galactosidase assay. However, results were comparable to the semi-quantitative assays (Fig. 3A and Table 1). Interestingly, R1699W was ~2-fold more active than the wild-type control. In conclusion, the contradiction found for variant R1699W was not due to a partial loss of function indistinguishable from the wild-type in semi-quantitative assays.

Figure 3. Transcriptional activity of BRCA1 variants. (A) Activity in yeast cells. The structure of the reporter plasmid is depicted above the graph. Variants (black bars) are in order of location in the structure of BRCA1 with the exception of the last three constructs (gray bars), which correspond to negative (M1775R and Y1853X) and positive (wild-type) controls. The shaded area represents a range of activity equal to or higher than wild-type. (B) Activity in human cells. The structure of the reporter plasmid is depicted above the graph. Variants (black bars) are in order of location in the structure of BRCA1 with the exception of the last three constructs (gray bars), which correspond to negative (M1775R and Y1853X) and positive (wild-type) controls. The shaded area represents a range of activity equal to or higher than wild-type. (C) Mutant R1699W is expressed at the same level as wild-type (gray arrow). The white arrow indicates expression of the GAL4 DBD moiety in the absence of any fusion fragment.
Analysis in mammalian cells

To further examine the transcription activity of the variants we performed assays in mammalian cells. With the exception of variants R1699W and R1699Q, transcription activation was comparable between yeast and mammalian cells (Fig. 3A and B). In 293T cells, variants R1699W and R1699Q displayed loss of function phenotype in accordance with pedigree analysis, suggesting that these variants are indeed cancer-associated mutations. Protein levels of R1699W and wild-type were similar, ruling out increased instability of the protein as the cause for the loss-of-function phenotype (Fig. 3C).

DISCUSSION

The notion that cancer-predisposing mutations in tumor-suppressor genes cause a loss-of-function phenotype is a key concept in cancer genetics. Here we utilized a functional assay to characterize clinically relevant BRCA1 variants. Our rationale was that transactivation activity of BRCA1 might mirror a functionally important feature of the protein in vivo and form the basis for a functional assay. Several lines of evidence have called attention to BRCA1 as a transcription regulator and it has been demonstrated that disease-associated mutations abolish the transactivation by BRCA1 in different experimental systems (for a review see ref. 9). Importantly, BRCA1 alleles carrying benign polymorphisms retain wild-type activity (24,26). Thus, relevant functional information might be gained from characterizing the effect of BRCA1 mutations on transcription activation. In addition, development of a functional assay for BRCA1 will fill a gap within the field directed at providing risk assessment information for counseling. The main difference between the present and past studies (24,25) is that this study is combined with pedigree and segregation analysis, providing a background to validate the results.

As demonstrated by our results in Table 1, the effect of an introduced BRCA1 mutation on transcription activation in the yeast-based assay is not affected by the DBD of the fusion protein or the promoter context of the reporter gene. Problems in interpreting results might nevertheless arise when characterizing variants that do not affect protein function in yeast. This is exemplified by the R1699 variants (Table 1). Although the clinical data indicate that R1699W is likely to predispose carriers to ovarian cancer (Fig. 2), our yeast-based tests revealed a wild-type activity, an apparent divergence between disease predisposition in vivo and the transcription activation assay (Table 1). This disagreement could not be explained by vector background or by differences in promoter stringency. However, we found that in the mammalian cell-based assay, transactivation activity of the R1699 variants was reduced in a fashion comparable to the negative controls. In fact, all variants presented here, with the exception of R1699W and R1699Q, behave similarly in the yeast- and mammalian-based assays. Thus, it is possible that specific protein alterations that have an effect on in vivo phenotype remain undetected in the simplified yeast model. We are currently investigating the reasons for this difference. Consequently, at this time we cannot unambiguously characterize variants that do not disrupt transcription activation in yeast as benign polymorphisms.

Using a mammalian-based assay to supplement results from the yeast assay might provide the scrutiny necessary to exclude or confirm disease predisposition of a certain variant. Similarly, mutations that affect mRNA processing in vivo might also be erroneously scored as a benign polymorphism because our assay is based on expression from an artificial cDNA. This could be the case for variant D1692N because the alteration affects a conserved guanine at a splice donor site and its potential effects on mRNA have not been examined. Conceivably, false negative results (i.e. benign polymorphisms that behave as loss-of-function mutants) can also occur when a particular variant causes message or protein instability in yeast. By extending our analysis using mammalian cells we should be able to distinguish those variants.

Considering the excellent correspondence between genetic alterations associated with breast and ovarian cancer in families and those that abolish transactivation, we tentatively characterized several additional BRCA1 unclassified variants. We propose that variants V1665M, D1692N and D1733G represent benign polymorphisms and variants V1668del and S1715N represent disease-associated mutations. Final characterization of these variants might await independent confirmation.

Our findings, taken together with previously published data (18,24,26), demonstrate a correlation between loss of transactivation activity and disease predisposition and it will be interesting to see whether future data will corroborate the predictions made here. Our results indicate that yeast-based assays can aid in the characterization of deleterious mutations in the C-terminal part of BRCA1 but it may be unable to unambiguously characterize benign polymorphisms. This is exemplified by mutations at residue R1699, for which we report a discrepancy in effect on transcription between yeast and mammalian cells. Thus, our study underlines the importance of analyzing the effect of putative disease-causing mutations in mammalian-based assays and taking into account data from population-based studies. In summary, we show that transcription activation may reflect the tumor-suppressing function of BRCA1 and provide further support for the role of missense mutations in disease predisposition.

MATERIALS AND METHODS

Yeast strains

Three S. cerevisiae strains were used: HF7c (MATa, ura3-52, his3-200, lys2-801, ade2-101, trp1-901, leu2-3 112, gal4-542, gal80-538, LYS2::GAL1-HIS3, URA3::(GAL1 17-mers)-CYC1-lacZ) (33); SFY526 (MATa, ura3-52, his3-200, ade2-101, lys 2-801, trp 1-901, leu 2-3, 112, canr, gal4-542, gal80-538, URA3::GAL1-lacZ (34); and EGY48 (MATa, ura3, trp1, his3, 6 lexA operator-LEU2) (35). HF7c and SFY526 contain reporter genes under the control of GAL1 UAS, which is recognized by GAL4 DBD. When activated, the reporter gene in SFY526 will produce β-galactosidase and HF7c will grow in minimal medium lacking histidine. EGY48 cells were transformed with plasmid reporters under control of LexA operators (pSH18-34, pJK103 or pRB1840) that produce β-galactosidase when activated (35).
Yeast expression constructs

A fusion construct containing GAL4 DBD:BRCA1 (amino acids 1560–1863) in pGBT9 (Clontech) used as a wild-type control and as a backbone to introduce mutations was described by Monteiro et al. (18). Specific alterations in BRCA1 were introduced by Quick-change site-directed mutagenesis (Stratagene) according to the manufacturer’s instructions. In short, primers containing the alteration were used in a PCR reaction to copy wild-type constructs produced in a methylation-competent bacterial strain and amplification was performed using *Pfu* polymerase. DpnI was subsequently added to digest the parental plasmid, leaving only cDNAs with introduced mutations to be transformed into bacteria. Confirmation of the introduced mutations was obtained by direct sequencing of the BRCA1 (amino acids 1560–1863) insert using two primers: GAL4 DNA-BD, 5′-TCATCGGAGA-GAGAGTAG-3′ (17-mer) (Clontech), and pGBT9 M13 REV, 5′-TGTTAAACGACGGCCCGTTTTTTTTAAAACCTAAGAGT-CAC-3′. For experiments in EGY48, BRCA1 inserts with mutations were subcloned into pLex9 (35) in-frame with the DBD of LexA. Both pGBT9 and pLex9 have *TRP1* as a selectable marker, allowing growth in medium lacking tryptophan.

Yeast transformation

Transformations were performed using the yeast transformation system based on lithium acetate (Clontech). Briefly, a single colony was inoculated in YPD medium for 16–18 h to produce a saturated culture. Cells were transferred to fresh medium and grown for 3 h, centrifuged, washed, resuspended in TE/LiAc (10 mM Tris–HCl, 1 mM EDTA, 100 mM lithium acetate, pH 7.5) solution and used immediately for transformation. Competent cells were incubated in polyethylene glycol (PEG)/LiAc (40% PEG 4000, 10 mM Tris–HCl, 1 mM EDTA, 100 mM lithium acetate, pH 7.5) solution at 30°C for 30 min with appropriate vector and carrier DNA. DMSO was added to 10% final concentration and the mix was heat shocked at 42°C for 15 min. Cells were subsequently chilled, centrifuged and resuspended in TE (10 mM Tris–HCl, 1 mM EDTA, pH 7.5). Cells were plated on synthetic dropout medium (SDM) and incubated at 30°C to select for transformants.

Yeast growth assay

Thirty-six individual HF7c clones for each variant were streaked on solid SDM lacking tryptophan and on SDM lacking both tryptophan and histidine and growth was scored after 2 days. A positive (+) or a negative (–) score was noted if growth was identical to the positive control (wild-type BRCA1 amino acids 1560–1863) or to the negative control (vector with no insert), respectively.

β-galactosidase assays

Thirty-six individual SFY526 clones and at least six individual EGY48 clones for every variant were streaked on filter paper overlaid on solid SDM lacking tryptophan (or tryptophan and uracil for EGY48). Plates were incubated for 2 days (SFY526) or 24 h (EGY48) and cells growing on the filter paper were lysed by freeze–thawing in liquid nitrogen and assayed for β-galactosidase activity in 2.5 ml of Z buffer (16 g/l *Na₂HPO₄*·7H₂O, 5.5 g/l *NaH₂PO₄*·H₂O, 0.75 g/l KCl, 0.246 g/l MgSO₄·7H₂O, pH 7.0) containing 40 μl of X-gal solution (20 mg/ml in *N*,*N*-dimethylformamide) and 6.6 μl of β-mercaptoethanol. For SFY526, a positive (+) or a negative (–) score was noted if the activity was identical to the positive control (wild-type BRCA1 amino acids 1560–1863) or to the negative control (vector with no insert), respectively. For EGY48 a positive score (+) was noted if the activity was identical to the positive control (wild-type BRCA1 amino acids 1560–1863) and a negative score (–) was noted if the activity was identical to the negative controls (M1775R and Y1853X). Clones were scored 6 h (SFY526) or 2 h (EGY48) after addition of X-gal. Liquid assays were performed as described by Brent and Ptashne (36). At least three separate transformants were assayed and each was performed in triplicate.

Transcription assay in mammalian cells

GAL4 DBD:BRCA1 fusions were subcloned into pCDNA3 (Invitrogen). We used pGSLuc, which contains a firefly luciferase gene under the control of five GAL4 binding sites (37). Transfections were normalized with an internal control, pRL-TK, which contains a *Renilla* luciferase gene under a constitutive TK basal promoter using a dual luciferase system (Promega). Human 293T cells were cultured in DMEM supplemented with 10% calf serum and plated in 24-well plates at ~60% confluence the day before transfection. Transfections were performed in triplicates using Fugene 6 (Roche) and harvested 24 h post-transfection.

Western blot

Cells were lysed in RIPA (150 mM NaCl, 10 mM Tris–Cl pH 7.4, 5mM EDTA, 0.1% sodium dodecyl sulfate, 1% Triton X-100, 0.1% sodium deoxycholate), boiled in sample buffer and separated on a 10% SDS–PAGE. Gels were electrophoresed on a wet apparatus to a polyvinylidene difluoride (PVDF) membrane. The blots were blocked overnight with 5% skim milk using TBS–TWEEN, and incubated with α-GAL4 DBD monoclonal antibody (Clontech) using 0.5% bovine serum albumin in TBS–TWEEN. The blots were subsequently incubated with the α-mouse IgG–horseradish peroxidase conjugate in 1% skim milk in TBS–TWEEN and developed using an enhanced chemiluminescent reagent (NEN).

Electronic database information

Online Mendelian Inheritance in Man (OMIM) is available at http://www.ncbi.nlm.nih.gov/omim. The Breast Cancer Information Core (BIC) is an online database of mutations in breast cancer susceptibility genes hosted by the National Human Genome Research Institute and can be accessed at http://www.ncbi.nlm.nih.gov/Intramural_research/Lab_transfer/Bic/.

ACKNOWLEDGEMENTS

We thank Niklas Dahl for providing information from family L321, Robert Coyne for sharing unpublished results and Pär-Ola Bendahl for help with statistics and presentation of data. This study was supported by the Swedish Cancer Society; the Lund Family American Cancer Society Grant, the Julia Murtha Fund, US Army award DAMD17-99-1-9389 and the following foundations: Mrs Berta Kamprads; Gunnar Arvid and...
Elisabeth Nilsson; Franke and Margareta Bergqvist; Hospital of Lund; King Gustav V’s Jubilee; Irving Weinstein; Lee Kaplan; and the AMDc Foundation of New York City.

REFERENCES


