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Award Number: DAMD17-01-1-0403

TITLE: Characterization and Use of Temperature-Sensitive Mutations of BRCA1 for the Study of BRCA1 Function

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REPORT DATE: July 2003

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	July 2003	Annual Summary (1 Jul 2002 - 30 Jun 2003)

4. TITLE AND SUBTITLE
Characterization and Use of Temperature-Sensitive Mutations of BRCA1 for the Study of BRCA1 Function

5. FUNDING NUMBERS
DAMD17-01-1-0403

6. AUTHOR(S)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
Original contains color plates: All DTIC reproductions will be in black and white.

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)
Recent evidence suggests that the breast and ovarian cancer susceptibility gene product BRCA1 is involved in at least two fundamental cellular processes: transcriptional regulation and DNA repair. The precise mechanism of action of BRCA1 in either of these processes is still unknown. We have identified a naturally-occurring allele of BRCA1 which codes for a single amino acid substitution from arginine to tryptophan at residue 1699 (R1699W). This mutation occurs in a region at the N-terminal BRCT domain that is highly conserved among BRCA1 homologs. When the C-terminus of the mutant protein (aa 1560-1863) was fused to a heterologous GAL4 DNA-binding domain and expressed in yeast or mammalian cells, it was able to activate transcription of a reporter gene to levels observed for wild type BRCA1 at the permissive temperature (30°C) but exhibited significantly less transcription activity at the restrictive temperature (37°C or 39°C). The transcription activity of the mutant protein appears to undergo complex regulation, as temperature-sensitive transcription activation was found to be cell type specific and not dependent on the tissue of origin. Stable cell lines expressing the mutant protein had similar capacity for DNA-damage repair of double strand breaks induced by ionizing radiation at all temperatures examined, suggesting that the activity of the mutant protein in DNA-damage repair is not temperature sensitive. . Our results demonstrate that the transcriptional activity of the R1699W) mutant can be modulated as a function of temperature and provide a novel experimental approach which can be utilized to dissect the molecular mechanism(s) of BRCA1 in processes related to transcription.

14. SUBJECT TERMS
Cancer biology, BRCA1, transcription activation, gene expression, DNA repair

15. NUMBER OF PAGES
23

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

INTRODUCTION

Mutations in the breast and ovarian cancer susceptibility gene *BRCA1* are observed in approximately 45% of families with a high incidence of breast cancer and 75% of families with multiple cases of breast and ovarian cancer (Easton et al., 1993; Struewing et. al., 1997). BRCA1 is involved in several cellular processes including transcription activation, cell cycle regulation, DNA-damage repair and maintenance of genomic stability (Monteiro, 2000; Venkitaraman, 2001). Alterations in BRCA1 may result in disruption of any or all of these processes and predispose to cancer.

BRCA1 is composed of 1863 amino acids and contains two BRCT domains present in tandem at the C-terminus of the protein. BRCT domains have been observed in several proteins involved in DNA-damage repair and cell cycle regulation (Koonin et. al., 1996; Bork et al., 1997; Callebaut and Mornon, 1997). When fused to a heterologous DNA-binding domain, the C-terminus of BRCA1 has been shown to activate transcription (Monteiro et. al., 1997; Monteiro et al., 1996; Chapman and Verma, 1996). Whereas cancer-associated missense mutations in the BRCT domains abolish this activity, benign polymorphisms do not (Vallon-Christersson et al., 2001)

As described in our original proposal, we have identified a cancer-predisposing temperature-sensitive mutation in the C-terminus of BRCA1. This missense mutation, R1699W, codes for a single amino acid substitution from arginine to tryptophan at residue 1699 and represents a naturally-occurring allele which was recently found to segregate with several cases of ovarian cancer in a Swedish family (Vallon-Christersson et. al., 2001). We observed that this mutation was able to activate transcription in both yeast and human embryonic kidney cells at the permissive temperature of 30°C. However, at 37°C the ability of this mutant to activate transcription was significantly decreased when compared to the activity of the wild-type. Based on these observations, we proposed that the activity of this mutant can be modulated as a function of temperature to more clearly define the mechanism(s) of BRCA1 in disease.

BODY

During the second year of the present grant we focused on the completion of Task 2 and have begun to work on Task 3:

Task 2. Establish and characterize stable cell lines (in HCC1937 cells) expressing the BRCA1 constructs created in Task 1 (months 2-26).

- Count the number of cells in growing cultures for each cell line at permissive and nonpermissive temperatures to obtain growth curves.
- Perform Western blots of lysates harvested above to assess protein expression levels.
- Perform immunohistochemistry on cultured cells to determine the intracellular localization of BRCA1 proteins.

Task 3. Use stable cell lines cultured at appropriate temperatures to assess BRCA1 function in DNA repair and protein-protein interactions (months 26-36).

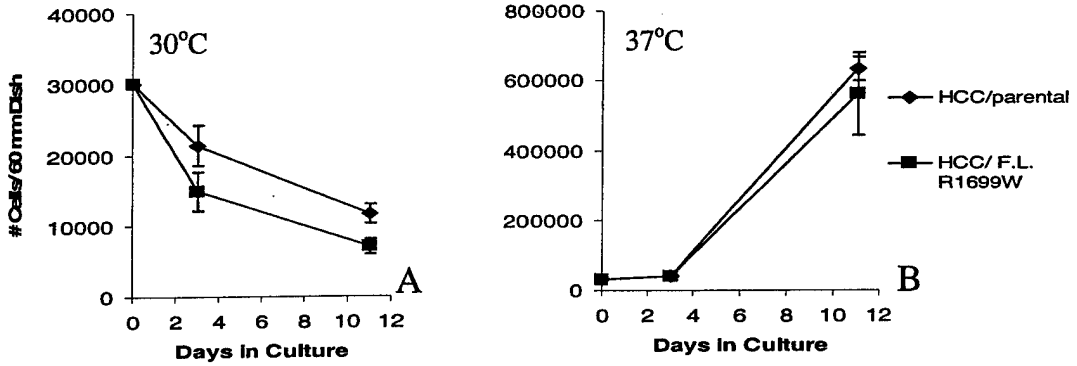
- Perform colony forming assays on cultures treated to specific doses of γ irradiation to assess double-strand DNA repair.
- Transiently transfect the mdm2 promoter reporter construct that has been damaged by UV irradiation for specific periods of time into cell lines and assay transcription activity to assess nick DNA repair

KEY RESEARCH ACCOMPLISHMENTS

Progress on goals defined in Task 2.

BRCA1-deficient HCC1937 and L56BR cells which stably express the R1699W mutation were characterized using western blot analysis and described in the DAMD17-01-1-0403 progress report 2001/2002. Western blot analysis of full length M1775R in stably transfected HCC1937 cells revealed similar expression levels as cells stably transfected with wild-type (data not shown). Stable cell lines of HCC1937 expressing Y1853X were not isolated despite several attempts. It was also reported in the DAMD17-01-0403 progress report 2001/2002 that human 293T and HCC1937 cells transiently transfected with full length R1699W and grown at the permissive temperature of 30°C poorly tolerated incubation periods longer than 36 hours. As was proposed in the original Statement of Work, we next assessed the ability of the stably transfected HCC1937 and L56BR cells to grow at permissive and nonpermissive temperatures. After 11 days in culture at the permissive temperature of 30°C, both parental and the stably-transfected cells were reduced to approximately one third of the original number plated (Fig. 1). Cells expressing the R1699W mutant were significantly more sensitive to the detrimental effects of growth at 30°C than were the untransfected parental cells (Fig. 1A). At 37°C, growth of both parental cells and cells expressing the R1699W full length mutant construct was unaffected (Fig. 1B). Our observations indicate that the increased transcription activity in the HCC/RW10.8B cells at the permissive temperature correlates with diminished survival of cells. This may be explained in part by the recent observation that ectopic overexpression of BRCA1 can trigger apoptosis (Thangaraju et al., 2000). Similar results at 30°C were obtained using L56BR cells that stably express the full length R1699W mutant construct (data not shown).

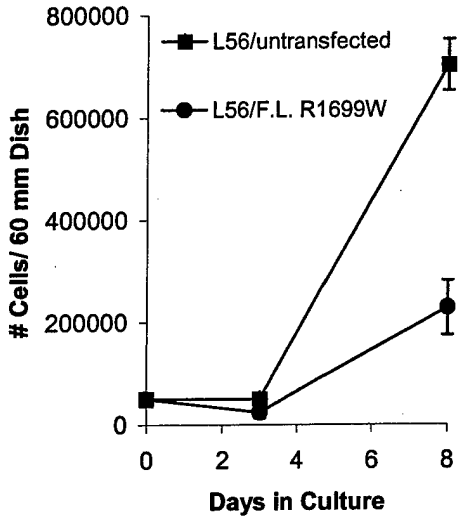
Figure 1: Effect of R1699W mutation on growth of HCC1937 cells.



HCC1937 parental and HCC/RW10.8B cells stably expressing the full length R1699W mutant construct of BRCA1 were plated onto 60 mm culture dishes and grown at 30°C (Panel A) or 37°C (Panel B). Data points represent the mean ± the standard deviation of triplicate samples.

We observed that L56BR cells expressing the R1699W mutant grow more slowly than the L56BR untransfected parental cells at 37°C (Fig. 2). This is of particular interest because this is the nonpermissive temperature observed for transcription activity in 293T and HCC1937 cells. The reduction in growth rate observed in R1699W-expressing L56BR cells when compared to the untransfected parental cells suggested that the R1699W mutant may possess residual activity at this temperature.

Figure 2: Effect of R1699W mutation on growth of L56BR cells



L56BR untransfected parental cells and L56BR/RW cells stably expressing the full length R1699W mutant construct of BRCA1 were plated onto 60 mm culture dishes and grown at 37°C. Data points represent the mean ± the standard deviation of triplicate samples.

We were unable to measure reporter gene expression in L56BR cells transfected with or without the R1699W fusion construct, possibly due to the low transfection efficiency of these cells. To explore the possibility that the R1699W mutation could have activity at 37°C, we analyzed the transcription activity as a function of temperature in several human-derived cancer cell lines and found that the R1699W mutant is regulated in a complex manner. These data were recently published (Worley et. al., 2002) and are attached in the Appendix. To briefly summarize, we found that the R1699W allele activated transcription at both 30° and 37°C in the ovarian cancer cell line Caov-2 and the breast cancer cell line MCF-7. In contrast, this allele was unable to activate transcription at either temperature in SKOV-3 ovarian cells. In NIH-OVCAR-3 ovarian cells, the R1699W allele acted similarly as in HCC1937 and 293T cells (Worley et. al., 2002). Taken together these data indicate that the temperature-sensitive transcription activity of the R1699W allele is cell-type dependent and not directly related to the tissue of origin.

Immunohistochemical analysis of BRCA1 expression using an antibody that recognizes a common epitope in the wild type and R1699W protein revealed that at both permissive and restrictive temperatures the respective proteins are predominately localized to the nucleus of HCC1937 cells (Fig. 3 and not shown). These data indicate that the cellular distribution of the full length mutant protein is normal.

Figure 3: Cellular localization of R1699W.

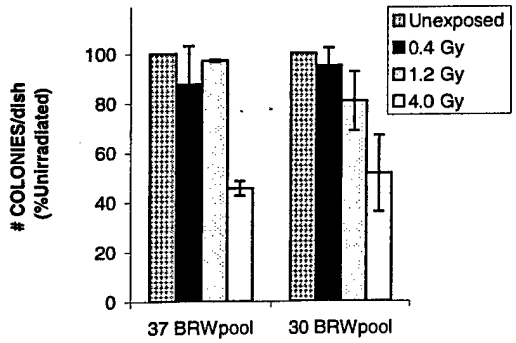
HCC1937 cells expressing the R1699W allele were cultured at 37°C, fixed and probed with BRCA1-specific antibody. The figure demonstrates that the R1699W protein is primarily nuclear.

To summarize, we have now thoroughly completed all of the components of Tasks 1 and 2. As mentioned above, we have recently published some of these data (Worley et. al., 2002) and this publication has been attached as Appendix A.

Progress on goals defined in Task 3.

In the DAMD17-01-0403 progress report 2001/2002 we described a mouse fibroblast cell line which was BRCA1 deficient and engineered to stably express the human R1699W allele. Using these cells, we examined the ability of the R1699W protein to mediate double strand break repair following DNA damage by radiation. Figure 4 depicts the ability of cells treated with or without increasing amounts of γ irradiation to recover and grow as colonies on cell culture dishes. 293T cells with wild-type BRCA1 levels served as the positive control. When compared to the BRCA1-proficient 293T cells, the mouse cells expressing the mutant protein (BRW) were significantly more resistant to doses of 1.2 and 4.0 Gy. Mouse cells showed no differences in DNA repair activity at either 30oC or 37oC. These data suggest that either the activity of the R1699W mutant protein at both temperatures is enough to mediate DNA repair or that the regulation of this protein is different in mouse cells than in 293T cells. We are currently exploring these options.

Figure 4: Effect of R1699W on colony forming ability following irradiation.



Cells were plated on 60 mm culture dishes at low density and on the following day exposed to increasing amounts of γ irradiation. Cells were then grown at 30°C or 37°C for 10 days. Bars represent the mean ± the standard deviation of triplicate samples.

To summarize, we have achieved the first goal of Task 3 as outlined in the original proposal and are on track to complete Task 3 in the alloted time. Nevertheless, we plan to use the R1699W conditional mutant to more closely study DNA damage repair. To this end, we have also established a system to study DNA-damage repair in irradiated cells using pulse-field gel electrophoresis.

ADDITIONAL ACHIEVEMENTS

As a complementary approach to using the temperature-sensitive mutants to modulate BRCA1 function, we have developed an expression plasmid coding for a short hairpin RNA that results in the breakdown of BRCA1 through RNA interference.

REPORTABLE OUTCOMES

- Development of human breast cancer cell lines expressing the R1699W mutant allele.
- Abstract and poster presentation entitled, "A Conditional Allele of BRCA1" at the DoD Era of Hope Meeting in Orlando, FL. September 2002 (P22-4).
- Manuscript entitled, "A Naturally-Occurring Allele of *BRCA1* Coding for a Temperature-Sensitive Mutant Protein." *Cancer Biology and Therapy*, **1:** 502-508. (DoD support is acknowledged; copy attached as appendix).
- Development of RNA intereference Expression Plasmid for BRCA1.
- Faculty Position Obtained in the Department of Pharmaceutical Sciences at St. John's University, Queens, NY

SUMMARY OF TASKS

Task 2. **Establish and characterize stable cell lines expressing BRCA1 constructs created in Task 1.**

We have completed all of the proposed experiments concerning this task as described in the original statement of work. Nevertheless, we will continue to use these cell lines as molecular tools to dissect the role of BRCA1 in cellular pathways pertaining to DNA damage and cell cycle control.

Task 3. **Use stable cell lines cultured at appropriate temperatures to asses BRCA1 function in DNA repair and protein-protein interactions.**

We have completed half of the proposed experiments concerning this third and final task as described in the original statement of work. We are on track to complete all proposed experiments in the allotted time frame.

CONCLUSIONS

We have begun to use the R1699W conditional mutant to assess the role of BRCA1 in cell growth and DNA-damage repair. As with the full length wild type protein, we have observed that this mutant protein is primarily localized to the nucleus. The regulation of transcription activation by the R1699W protein appears to be cell-type specific. 293T, HCC1937 and NIH-OVCAR-3 cells display temperature-sensitivity with respect to transcription activation by the R1699W protein. Prolonged growth of R1699W-expressing cells at the permissive temperature of 30°C was detrimental to cell viability. Of particular interest was the observation that whereas transcription activation of the R1699W mutant was dependent on temperature, the DNA-damage repair activity of this mutant was temperature independent. The uncoupling of transcription activity from DNA damage repair using the R1699W mutant protein was an unexpected finding that will enable closer study of the role of BRCA1 in transcription related pathways. Using RNA interference directed against BRCA1, we plan to validate our observations obtained with the temperature-sensitive R1699W and the other previously described H1686Q mutant with respect to transcription. We also plan to examine the effect of the R1699W mutation on cell cycle checkpoint regulation.

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[Cancer Biology & Therapy 1:5, 497-501, September/October 2002]; ©2002 Landes Bioscience

Research Paper

A Naturally Occurring Allele of BRCA1 Coding for a Temperature-Sensitive Mutant Protein

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Received 8/25/02; Accepted 8/30/02

Previously published online as a CB&T "Paper in Press" at:
http://landesbioscience.com/journals/cancerbio/papersinpress/inpress15.html

KEY WORDS

BRCA1, Yeast, Transcription, Temperature-sensitive mutants, BRCT domain, Ovarian cancer

This work was supported by NIH 1R01 CA92309 (A.M.), DoD awards DAMD17-99-1-9389 (A.M.) and DAMD17-01-1-0403 (B.B.), the Swedish Cancer Society, the King Gustav V's Jubilee foundation, and the American Cancer Society (A.B.).

ABSTRACT

Recent evidence suggests that the breast and ovarian cancer susceptibility gene product BRCA1 is involved in at least two fundamental cellular processes: transcriptional regulation and DNA repair. However, the mechanism of action of BRCA1 in either of these processes is still unknown. Here, we report the characterization of a disease-predisposing allele of BRCA1, identified in a family with several cases of ovarian cancer, coding for a protein that displays temperature-sensitive activity in transcriptional activation. The mutant protein differs from the wild type protein at a single amino acid, R1699W that occurs in a region at the N-terminal BRCT domain that is highly conserved among BRCA1 homologs. When the C-terminus of the mutant protein (aa 1560–1863) was fused to a heterologous GAL4 DNA-binding domain and expressed in yeast or mammalian cells, it was able to activate transcription of a reporter gene to levels observed for wild type BRCA1 at the permissive temperature (30°C) but exhibited significantly less transcription activity at the restrictive temperature (37°C or 39°C). Our results indicate that the transcriptional activity of the R1699W mutant can be modulated as a function of temperature and provide a novel experimental approach which can be utilized to dissect the molecular mechanism(s) of BRCA1 in processes related to transcription.

INTRODUCTION

Individuals carrying inactivating germline mutations in BRCA1 (OMIM 113705) have an increased predisposition to breast and ovarian cancer.[1,2] A growing body of evidence indicates that the breast and ovarian cancer susceptibility gene product BRCA1 is involved in at least two fundamental cellular processes: transcriptional regulation and DNA repair.[3-5] However, the mechanism of action of BRCA1 in either of these processes is still unknown.

During our analysis of BRCA1 germline missense mutations we came across a naturally occurring BRCA1 allele, identified in a family with several cases of ovarian cancer in which the mutation segregates with disease.[6] This allele carries a single point mutation (nucleotide C5214T), leading to a change from an arginine to a tryptophan residue at position 1699, located at the C-terminal region of BRCA1. The BRCA1 C-terminus (aa 1560–1863) has the ability to activate transcription when fused to a heterologous DNA binding domain (DBD) and introduction of disease-associated germ-line mutations impair transcription activation, while benign polymorphisms do not.[6-10] Missense mutations in BRCA1 that abolish transcription activation or disrupt the N-terminal RING finger structure also affect the ability of BRCA1 to interact with the RNA polymerase II holoenzyme in vitro and in vivo.[11,12] The C-terminal region encompasses two BRCT domains in tandem (BRCT-N [aa 1649–1736]; BRCT-C [aa 1756–1855])[13-15] and disruption of these domains is linked to cancer susceptibility. Interestingly, the ability to activate transcription does not seem to be a general characteristic of BRCT domains since BRCT domains isolated from other proteins, with the exception of RAP1, do not possess such activity.[16]

We previously observed that the R1699W mutant retains wild-type transcriptional activity in yeast but displays a loss-of-function phenotype when transcription activity is assessed in human cells.[6] Considering the conservation of basal transcription machinery in yeast and human cells,[17] we hypothesized that the discrepancy in transcription activity was due to differences at the temperature in which the cells were being cultured; 30°C for yeast cells and 37°C for human cells. In the present study we demonstrate that the cancer-predisposing R1699W variant of BRCA1 acts as a temperature-sensitive mutant in both yeast and human cells in transcription activation assays.

MATERIALS AND METHODS

Yeast. *Saccharomyces cerevisiae* strain EGY48 [*MATα, ura3, trp1, his3*, 6 lexA operator-*LEU2*] contains a LexA-responsive *LEU2* gene, which when activated permits growth in the absence of leucine.[18] Transformations were performed using the yeast transformation system based on lithium acetate (Clontech) according to the manufacturer's instructions.

Yeast Expression Constructs. Constructs containing the fusion GAL4 DBD:BRCA1 wild type (amino acids 1560–1863) or mutants R1699W, M1775R and Y1853X were previously described.[6] BRCA1 inserts (wild type and mutants) were subcloned into pLex9 in-frame with the DBD of LexA. A *TRP1* selectable marker is present in pLex9, allowing growth in medium lacking tryptophan.

Yeast Growth Assay. Cells were transformed with the LexA DBD fusion constructs and plated in solid medium lacking tryptophan. At least three independent colonies for each construct were inoculated into liquid medium lacking tryptophan and grown to saturation (OD_{600} ~1.5) at 30°C. Saturated cultures were used to inoculate fresh medium lacking tryptophan or medium lacking tryptophan and leucine to an initial OD_{600} of 0.0002. Parallel cultures were then incubated at 30°C or 37°C and growth was assessed by measurement at OD_{600} after 38 hr.

Transcription Assay in Mammalian Cells. The region comprising the GAL4 DBD fused to BRCA1 C-terminus containing the R1699W mutation was excised from pGBT9 backbone[6] with *Hind*III and *Bam*H1 and ligated into pCDNA3. Constructs in pCDNA3 containing fusion of GAL4 DBD and wild type BRCA1, M1775R or Y1853X variants were previously described.[6] We used the reporter pG5E1bLuc, which contains a firefly luciferase gene under the control of five GAL4 binding sites[19] and 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 5% calf serum and plated in 24-well plates at ~60% confluence the day before transfection. Transfections were carried out in triplicates using Fugene 6 (Roche, Indianapolis, IN) at 37°C for 12 hr. Cells were then incubated at 30°C, 37°C or 39°C and harvested 16 hr later. Human cell lines NIH-OVCAR-3 and SKOV-3, both derived from ovarian adenocarcinoma, and CAOV-2, derived from the malignant ascites of a patient with progressive adenocarcinoma of the ovary, were kindly provided by Jeff Boyd (Memorial Sloan Kettering).

Western Blotting. Yeast cells were grown in selective media to saturation and OD_{600} was measured. Cells were harvested and lysed in cracking buffer (8M Urea; 5% SDS; 40 mM Tris-HCL [pH6.8]; 0.1 mM EDTA; 0.4 mg/ml bromophenol blue; use 100 ml per 7.5 total OD_{600}) containing protease inhibitors. The samples were boiled and separated on a 10% SDS-PAGE. The gel was electroblotted on a wet apparatus to a PVDF membrane. The blots were blocked overnight with 5% skim milk using TBS-tween, and incubated with the a-pLexA (for LexA constructs) monoclonal antibody (Clontech) using 0.5% BSA in TBS-tween. After four washes, the blot was incubated with the a-mouse IgG horseradish peroxidase conjugate in 1% skim milk in TBS-tween. The blots were developed using an enhanced chemiluminescent reagent (NEN, Boston, MA).

RESULTS

A Temperature-Sensitive Phenotype in Yeast. We previously observed that the R1699W mutant (Fig. 1) retains wild-type transcriptional activity in yeast but displays a loss-of-function phenotype when transcription activity is assessed in human cells.[6] This discrepancy was not due to differential protein stability, vector background or promoter stringency in the reporter.[6] To further investigate this phenomenon, we generated yeast cell lines with an inducible GAL4 DBD: R1699W BRCA1 fusion integrated in the yeast genome as a single copy. In this context, the R1699W variant also displays activity comparable to wild type BRCA1, ruling out the possibility that the results were due to abnormally high levels of the protein expressed by an episomal plasmid (results not shown).

We then reasoned that differences in transcription activation could be due to differences in the temperature at which the cells were being cultured; 30°C for yeast cells and 37°C for human cells. To test this idea directly, we transformed *Saccharomyces cerevisiae* EGY48 with cDNAs coding for fusions of LexA (DBD) and the wild type C-terminal region of BRCA1 (aa 1560–1863) or constructs carrying either the R1699W mutant or one of two other germline disease-associated mutations, M1775R and Y1863X, as negative controls. Transcription activity was quantified at 30°C and 37°C using an integrated reporter gene (6 *lexA* binding sites; *LEU2*) that, when activated, allows growth in the absence of leucine. Cells carrying the wild-type BRCA1 construct were able to grow in selective medium lacking leucine at both temperatures (Fig. 2A). Conversely, cells carrying the two disease associated mutants did not show any detectable growth at either temperature (Fig. 2A). Interestingly, cells carrying the R1699W mutant were able to grow at levels comparable to the wild type at 30°C but growth was dramatically impaired at 37°C, indicating a marked reduction in transcriptional activity (Fig. 2A). Expression was comparable for the R1699W and the wild type protein at both temperatures (Fig. 2B).

The R1699W Variant Displays Temperature-Dependent Activity in Human Cells. To confirm the temperature-dependent activity of the R1699W variant, we cotransfected human kidney 293T cells with a luciferase reporter gene driven by GAL4-responsive promoter and cDNAs coding for fusions of GAL4 DBD with the wild type C-terminal region of BRCA1 (aa 1560-1863), the R1699W mutant or either of the two disease-associated mutations, M1775R and Y1863X, as negative controls. We then incubated cells in parallel at two temperatures: 30°C and 37°C. The disease-associated mutants M1775R and Y1853X display a small increase in relative activity at 30°C but their activity is significantly lower than the wild type BRCA1 (Fig. 3A, left panel). Interestingly, the transcriptional activity of the R1699W mutant was restored to wild type levels when cells were cultured at 30°C, indicating that this mutant acted as a temperature-sensitive allele of BRCA1 in transcription activation (Fig. 3A, left panel). Although at 37°C the R1699W mutant displays residual activity, experiments conducted at 39°C indicated a further reduction in activity (Fig. 3A, right panel). Expression was comparable for R1699W, M1775R and the wild type protein at both temperatures (not shown).

A Complex Regulation in Breast and Ovarian Cancer Cells. Considering the occurrence of multiple ovarian cancers (but not breast cancer) in the family in which the R1699W was identified, Lund 279, we next asked whether ovarian cancer cell lines were different from breast cancer cell lines with respect to the temperature-sensitive phenotype. We tested transcriptional activation in two

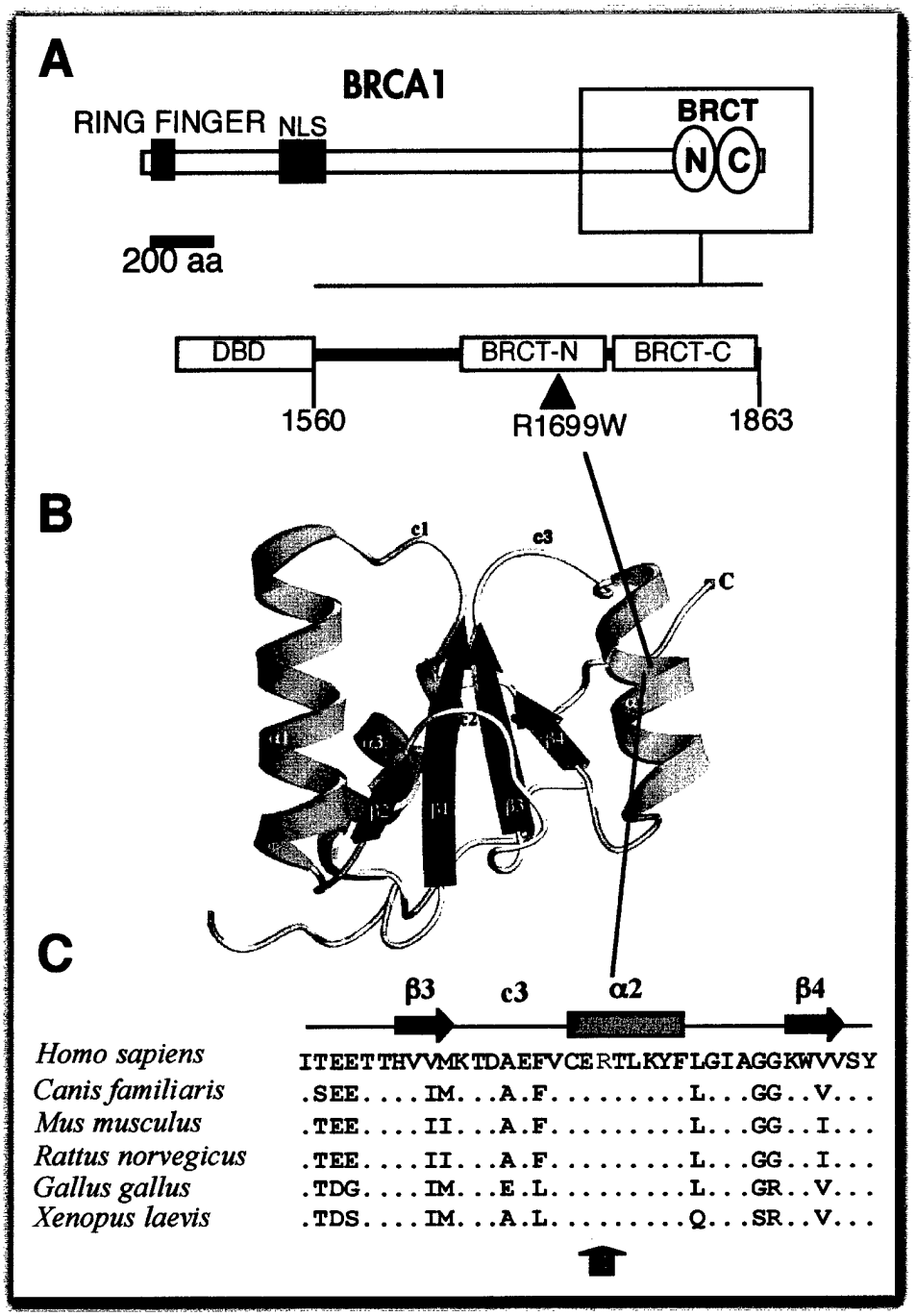

Figure 1. A temperature-sensitive BRCA1 mutant in transcription activation. (A) Top panel. Schematic representation of full length BRCA1 protein featuring: the RING domain in the N-terminus; the BRCT domains in the C-terminus (gray circles) and the nuclear localization signals (NLS). The region analyzed in this study is contained in the box, which is enlarged and represented in the bottom panel. Bottom panel. GAL4- and LexA-DNA binding domain (DBD) fusions to BRCA1 C-terminus (aa 1560–1863). The location of the R1699W mutation is indicated by a filled triangle. (B) Model of three-dimensional structure of BRCA1 BRCT (from ref. 30 by permission from Oxford University Press) indicating the location of the R1699 residue. (C) Alignment of BRCA1 homologs with secondary structures indicated on top. Dots represent identical amino acids. Location of R1699 residue is indicated with a red arrow.

breast cancer cell lines (MCF-7 and HCC1937) and three ovarian cancer cell lines (OVCAR-3, CaOV-2 and SKOV-3)(Fig. 3B). Surprisingly, the experiments revealed a complex regulation of this mutant in different human cancer cell lines. In MCF-7 and Caov-2, the R1699W allele was able to activate transcription to levels comparable to wild type BRCA1 at permissive and restrictive temperatures (Fig. 3B). In HCC1937 and NIH-OVCAR-3, the R1699W displayed temperature-dependent activity with normal activity at 30°C and loss of function at 37°C, consistent with our previous observation in 293T cells (Fig. 3A, B). Interestingly, in SKOV-3 cells the R1699W displayed a loss-of-function phenotype at both temperatures (Fig. 3B). In conclusion, our results did not reveal any correlation of the temperature-sensitive phenotype and tissue of origin and suggest that the R1699W variant may have a cell-type specific temperature-sensitive phenotype.

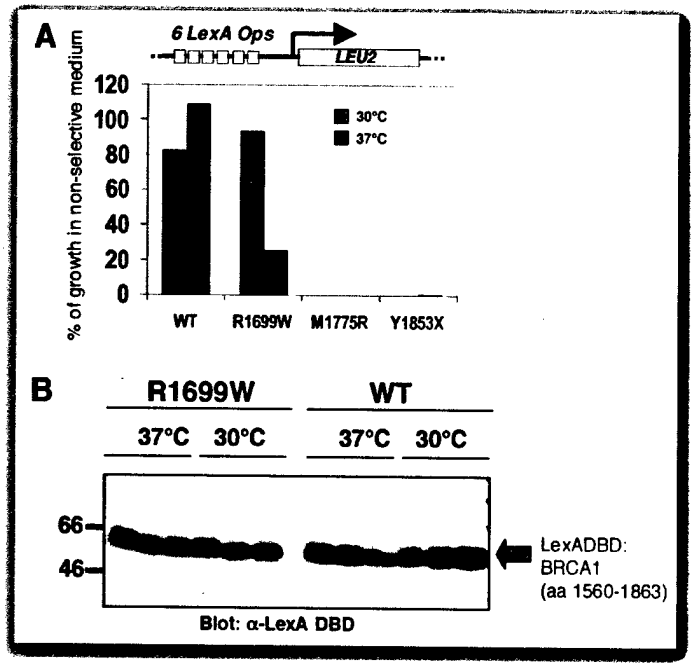

Figure 2. Transcriptional activity of BRCA1 R1699W at different temperatures in yeast. (A) Activity in yeast cells as measured by activation of an integrated LEU2 gene. Cells were cultured in non-selective and selective medium (lacking leucine) at 30°C and 37°C for 38 hr. Growth was measured by OD_{600}. Growth in non-selective medium was considered 100%. M1775R and Y1853X mutants were used as negative controls. (B) Mutant R1699W is expressed in yeast at the same level as wild type (gray arrow) at both temperatures. Three independent clones are shown for each condition. Blot was probed with α-LexA DBD monoclonal antibody.

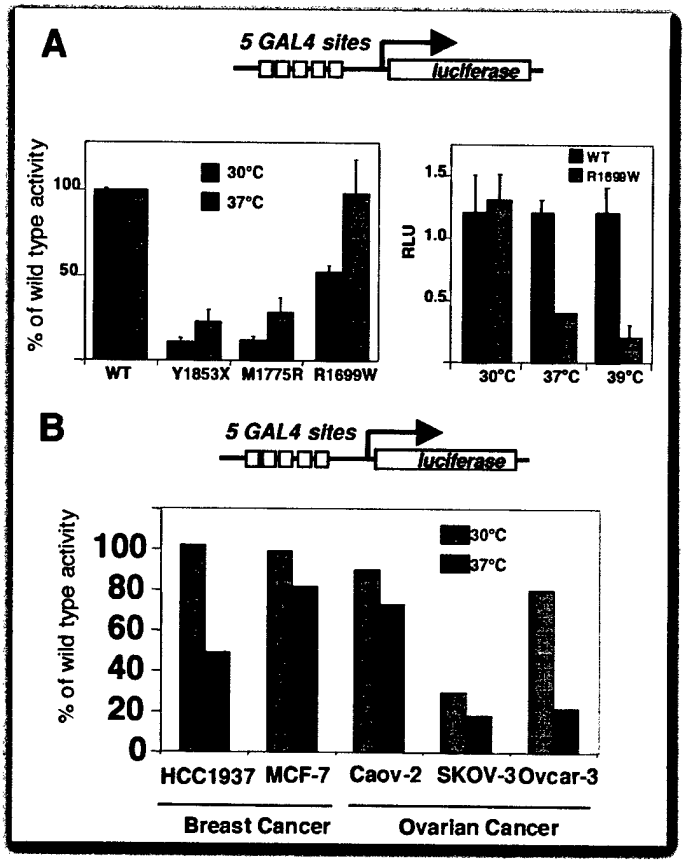

Figure 3. Transcriptional activity of BRCA1 R1699W at different temperatures in human cells. (A) Left panel. Transcriptional activity of the wild type BRCA1, R1699W, Y1853X and M1775R mutants at 30°C (gray bars) and 37°C (black bars). Activity of the R1699W variant in 293T cells is comparable to wild type at 30°C but markedly reduced at 37°C. Right panel. Transcriptional activity of the wild type BRCA1 and the R1699W mutant at different temperatures. RLU, relative luciferase units. Represents the ratio between Firefly luciferase and Renilla luciferase (internal control). (B) Temperature-sensitive activity of R1699W is cell type-specific. Average of three independent experiments. HCC1937, MCF-7 are breast cancer cell lines; CaOV-2, SKOV-3, NIH-OVCAR-3 are ovarian cancer cell lines. Structures of the reporters are depicted on top of the graphs. Transfections are normalized with a constitutive Renilla luciferase reporter.

DISCUSSION

The biochemical function of BRCA1 has remained elusive and the current evidence suggests that BRCA1 may have a pleiotropic function in the DNA damage response pathway and may be able to influence several activities that revolve around DNA damage resolution.[3,5,20-23] Alternatively, BRCA1 being a large multifunctional protein may have a wide range of unrelated biochemical activities in the cell. One approach to understand the function of a protein with tumor suppressor action is to analyze naturally occurring mutations that cause cancer predisposition.

In our study of missense mutations found in individuals with high risk for breast and ovarian cancer we came across a naturally occurring BRCA1 allele identified in a family from Lund that displayed unusual behavior in the transcription activation assay.[6] The clinical data suggests that the R1699W mutation (Arg to Trp substitution at codon 1699; see also Breast Cancer Information Core Database at http://research.nhgri.nih.gov/bic/) is likely to have a deleterious effect in vivo and predispose carriers to cancer. Disease association is further emphasized by the presence of the R1699W mutation in a large pedigree with several women diagnosed with ovarian cancer (Tom Frank, personal communication). Nonetheless, our initial transcription-based tests of this variant showed that it retained wild type activity in yeast but not in mammalian cells.[6] Previous studies had shown a complete concordance between results in yeast and mammalian cells.[7,8,24] This apparent divergence could not be explained by vector background, promoter stringency or abnormally high levels of the protein expressed by an episomal plasmid but rather was due to temperature differences at which the assays were carried out. Our results demonstrate that the R1699W variant display a temperature-sensitive phenotype in transcription activation and therefore may represent the first conditional mutant of BRCA1 to be described (Fig. 2).

The R1699W mutation is located in the BRCT domain, a region that is involved in binding to many different proteins that associate with BRCA1[3] and crucial for transcriptional activity.[10] The mutation, R1699W, replaces an arginine residue involved in a salt bridge that is thought to stabilize the packing of the two BRCT domains.[25] It occurs in a region at the N-terminal BRCT domain that is highly conserved among BRCA1 homologues (Fig. 1C). Interestingly, this region is not found in other BRCT motifs and seems to be unique to BRCA1 BRCT.[14,15] In fact, the predicted α-helix 2, in which the mutation resides, is conserved in all known BRCA1 homologs (Fig. 1C) and has been proposed to be responsible for determining functional specificity of the BRCT domains.[26]

Interestingly, Lund 279 presents almost exclusively ovarian cancer cases and no breast cancer cases raising the possibility of

differential effects of this mutant in breast versus ovary epithelia. That observation led us to test the temperature-sensitiveness in breast cancer and ovarian cancer cell lines. Intriguingly, our results indicate that the temperature-sensitive phenotype of the R1699W mutant is cell type specific but no tissue correlation was apparent with the cell lines tested (Fig. 3). At this point we can only speculate on the nature of such behavior and propose the following possible scenarios:

1. Residue 1699 is involved in binding to a factor required for transcription activation by BRCA1. The mutation would cause a marked decrease in binding affinity that is less severe at lower temperatures, therefore making it susceptible to variations in the concentration of this factor. For example, in cells where the factor is abundant, the R1699W variant would still be able to bind enough of the factor to promote transcription at both temperatures. Conversely, in cells in which the factor was at very low concentrations the R1699W variant would not be able to recruit the factor at either temperature. At intermediate concentrations of the factor, the R1699W variant would be able to bind it at 30°C but its ability to recruit the factor would be extremely reduced at 37°C.
2. Alternatively, it is also possible that instead of required for transcription, the function of this factor is to confer stability to the mutated protein (e.g., chaperone). In this case, the scenario of varying concentrations described above, or differential expression would also be applicable. This scenario is consistent with the observed increase in activity of the other mutants at 30°C (Fig. 2C).

Recently, mutants of the Xeroderma pigmentosum group D (XPD) helicase subunit of TFIIH displaying a temperature-sensitive phenotype in transcription and DNA repair have been isolated from patients with trichothiodystrophy (TTD). Patients carrying the mutant allele have a fever-dependent reversible deterioration of TTD features.[27,28] In this case, the phenotype manifests predominantly in the skin, hair and nails for reasons that are not well understood.[27] This finding raises important questions about the implications of the R1699W variant to the clinical phenotype. It is possible that small differences in temperature between breast and ovary may be responsible for an increased incidence of ovarian cancer. It remains to be seen if other families carrying this allele also display a preferential occurrence of ovarian cancer. These data also suggest that conditional mutants of BRCA1 in transcription may also have a conditional phenotype in the DNA damage response. We are currently exploring these possibilities.

In parallel with the present study we performed a random mutagenesis screen in yeast and identified 11 additional temperature-sensitive mutants of BRCA1.[29] Different from the variant described in this study, R1699W, which is an exposed surface residue, these additional TS mutants localized primarily to the hydrophobic core of the BRCT-N domain of BRCA1.[29] Further characterization is needed to assess whether these conditional mutations of BRCA1 may serve as experimental tools to dissect the precise molecular role of BRCA1 in processes related to transcriptional regulation.

Acknowledgements

The authors wish to thank Jeff Boyd for providing ovarian cancer cell lines and Marcelo Carvalho for helpful discussions.

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Research Article

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[Cancer Biology & Therapy 1:5, 502-508, September/October 2002]; ©2002 Landes Bioscience

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Received 4/23/02; Accepted on 7/22/02

Previously published online as a CB&T "Paper in Press" at: http://landesbioscience.com/journals/cancerbio/papersinpress/inpress15.html

KEY WORDS

BRCA1, Yeast, Yranscription, Temperature-sensitive mutants, BRCT domain

This work was carried out with support from NIH 1R01 CA92309 (A.N.A.M.), U.S. Army awards DAMD17-99-1-9389 (A.N.A.M.) and DAMD17-01-1-0403 (B.B.), a CAPES fellowship (M.A.C.) and the Fashion Footwear Association of New York/QVC.

ABSTRACT

BRCA1 is a tumor suppressor gene and germ line mutations account for the majority of familial cases of breast and ovarian cancer. There is mounting evidence that BRCA1 functions in DNA repair and transcriptional regulation. A major hurdle to dissect the role of BRCA1 is the lack of molecular reagents to carry out biochemical and genetic experiments. Therefore, we used random mutagenesis of the C-terminus of BRCA1 (aa 1560–1863) to generate temperature-sensitive (TS) mutants in transcription activation. We obtained 11 TS mutants in transcription that localized primarily to the hydrophobic core of the BRCT-N domain of BRCA1. One of the mutants, H1686Q, also displayed temperature-dependent transcription activation in human cells. These conditional mutants represent valuable tools to assess the role of BRCA1 in transcription activation.

INTRODUCTION

Germ-line mutations in BRCA1 confer high risk for breast and ovarian cancer.[1,2] The molecular function of BRCA1 is not yet known but there is increasing evidence that it is involved in DNA damage repair and gene transcription.[3,4] Several lines of evidence support a direct role for BRCA1 in transcription. When fused to a heterologous DNA binding domain (DBD) the C-terminus of BRCA1 activates transcription from a reporter gene and the introduction of cancer-associated mutations, but not benign polymorphisms, abolish[25] activation.[5-7] In addition, BRCA1 interacts with the RNA polymerase II and with several complexes involved in chromatin remodeling.[8-11] Ectopic expression of BRCA1 results in the transcription of genes involved in cell cycle control and DNA damage repair.[12-17] Interestingly, BRCA1 also interacts with CsTF50 in a complex that regulates mRNA processing pointing to a pleiotropic role in transcription.[18]

Despite the absence of BRCA1 homologs in its genome, yeast has been an important model system to study BRCA1 as well as the function of several mammalian transcription factors.[19] Yeast has been utilized to perform structure-function analysis of BRCA1 in transcription as well as to probe its mechanisms of activation based on the correlation with the clinical data.[5,7,20-22] In addition, overexpression of human BRCA1 in yeast generates a small colony phenotype that has been proposed as a method to classify uncharacterized mutations in BRCA1.[23,24] Thus, despite its limitations, yeast is a defined system to analyze BRCA1 function and is adequate for the rapid screening of large mutant libraries.

A major hurdle to define the function(s) of BRCA1 is the lack of molecular tools. Temperature-sensitive (TS) mutants would be particularly useful for this analysis. Recently, we have identified a BRCA1 allele in a family with familial ovarian cancer that displays a temperature-sensitive phenotype in mammalian cells (refs. 21, 41). Therefore, we hypothesized that a differential screen in yeast based on random mutagenesis would allow us to isolate additional TS mutants. We followed the same procedure we had previously used to generate loss-of-function mutants in transcription activation and performed parallel screens at 30°C and 37°C.[20] We utilized this yeast-based system to identify and characterize 11 TS mutations and 15 loss-of-function (LF) mutants of BRCA1. One of the TS mutations identified in the yeast screen was found to exhibit a similar phenotype in human cells. These mutants will allow the study of BRCA1 function in yeast and provide a basis for the development of novel conditional mutants for mammalian cells.

METHODS

Yeast. *Saccharomyces cerevisiae* strain EGY48 [*MATα, ura3, trp1, his3, 6 lexA* operator-*LEU2*] was co-transformed with the LexA fusion vectors and reporter plasmid pSH18–34, which has lacZ under the control of 8 LexA operators.[25,26] The LexA DBD fusion of wild type human BRCA1 C-terminus

(aa 1560–1863) and two germ-line mutants of BRCA1, Y1853X and M1775R were used as controls. Competent yeast cells were obtained using the yeast transformation system (Clontech).

Error-Prone PCR Mutagenesis and Screening. A 30-cycle PCR reaction (94°C denaturation; 55°C annealing; 72°C extension) was performed using *Taq* polymerase, p385-BRCA1 plasmid as a template and oligonucleotide primers (S9503101, 5′-CGGAATTCGAGGGAACCCCTTACCTG-3′; S9503098, 5′-GCGGATCCGTAGTGGCTGTGGGGGAT-3′). PCR products were gel purified and co-transformed in an equimolar ratio with an *NcoI*-linearized wild-type pLex9 BRCA1 (aa 1560–1863) plasmid and pSH18-34. Transformants carrying the mutagenized cDNAs were plated at 37°C or 30°C on plates lacking tryptophan and uracil and containing 80 mg/L X-Gal (5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside), 2% Galactose, 1% Raffinose, 1X BU salts (1L of 10X BU salts: 70g $Na_2HPO_4.7H_2O$, 30g NaH_2PO_4). The X-gal plates allowed direct visualization and were scored after 6 days. Clones were recovered from yeast and sequenced.

Mammalian Cell Reagents. A region comprising the BRCA1 coding region containing the TS mutation in pLex9 vector was excised with *EcoR*1 and *Bam*H1 and subcloned in pGBT9 in frame with GAL4 DBD. The fusion GAL4 DBD: BRCA1 was then cut with *Hind*III and *Bam*H1 and ligated into pCDNA3. We used the reporter pG5E1bLuc, which contains a firefly luciferase gene under the control of five GAL4 binding sites and transfections were normalized using a dual luciferase system (Promega). For the mammalian two-hybrid system the pCDNA3 GAL4 DBD: BRCA1 (aa 1560–1863) and the constructs carrying different TS mutations were used as bait to test interaction against CtIP. The construct containing CtIP (aa 45–897) fused to the herpesvirus VP16 transactivation domain (aa 411–456) was used as target and the VP16 vector was used as negative control (gift from Richard Baer, Columbia University). Human 293T cells were cultured in DMEM supplemented with 5% calf serum and plated in 24-well plates at ~60% confluence the day before transfection. Transfections were carried out in quadruplicates using Fugene 6 (Roche, Indianapolis, IN) at 37°C for 12 hr. Cells were then incubated at 30°C or 37°C and harvested 16 hr later.

RESULTS

Screen for TS Mutants of BRCA1 in Transcription Activation. We screened ~ 3 x 10^6 independent clones and recovered 1,302 putative LF mutants at 37°C (Fig. 1A). These colonies were then plated on fresh plates and incubated at 37°C and 30°C for confirmation (Fig. 1B). All plates contained yeast expressing wild-type cDNA to control for the different activity of β-galactosidase at both temperatures. Several clones turned out to display either a loss-of-function (white clones) or wild-type (blue clones) phenotype at both temperatures. Plasmids were recovered, retransformed into yeast and their activity confirmed. Clones that failed to display a reproducible activity were discarded. Plasmids representing 38 clones (3 were not recovered) were analyzed by restriction digest and although no clone had detectable deletions/insertions by gel analysis, sequencing revealed that 12 had nucleotide deletions or nonsense mutations and were not analyzed further. The remaining clones were processed for sequencing and the mutation identified. Eleven clones displayed markedly reduced activity at 37°C and wild-type activity at 30°C (TS clones; Table 1) and 15 had reduced activity at both temperatures (LF clones, Table 2).

TS Mutants in Yeast. Our screen resulted in the isolation of 11 TS mutants (8 unique) in transcription activation in yeast (Table 1). Seven clones displayed only one missense mutation and four clones displayed two missense mutations (Table 1). It is unclear whether the two mutations are required for the TS phenotype or not. At least in one case, TS32 (S1722F/K1667E), we know this is not the case because a similar mutation was found independently in another clone, TS25. Mutations causing TS activity were found in exons 16–20 and 24. Interestingly, conserved hydrophobic residues were found to be a major target of mutations followed by mutations in serine residues (Table 1 and Fig. 2). With three exceptions, S1631N, L1639S and E1836G, all mutations occurred either in the N-terminal BRCT region or in the interval between the N- and C-terminal BRCTs (Table 1 and Fig. 2).

Loss-of-Function (LF) Mutants. Due to the experimental design, several clones proved not to be TS mutants but instead LF mutants at both temperatures tested (Table 2). These mutations also targeted hydrophobic

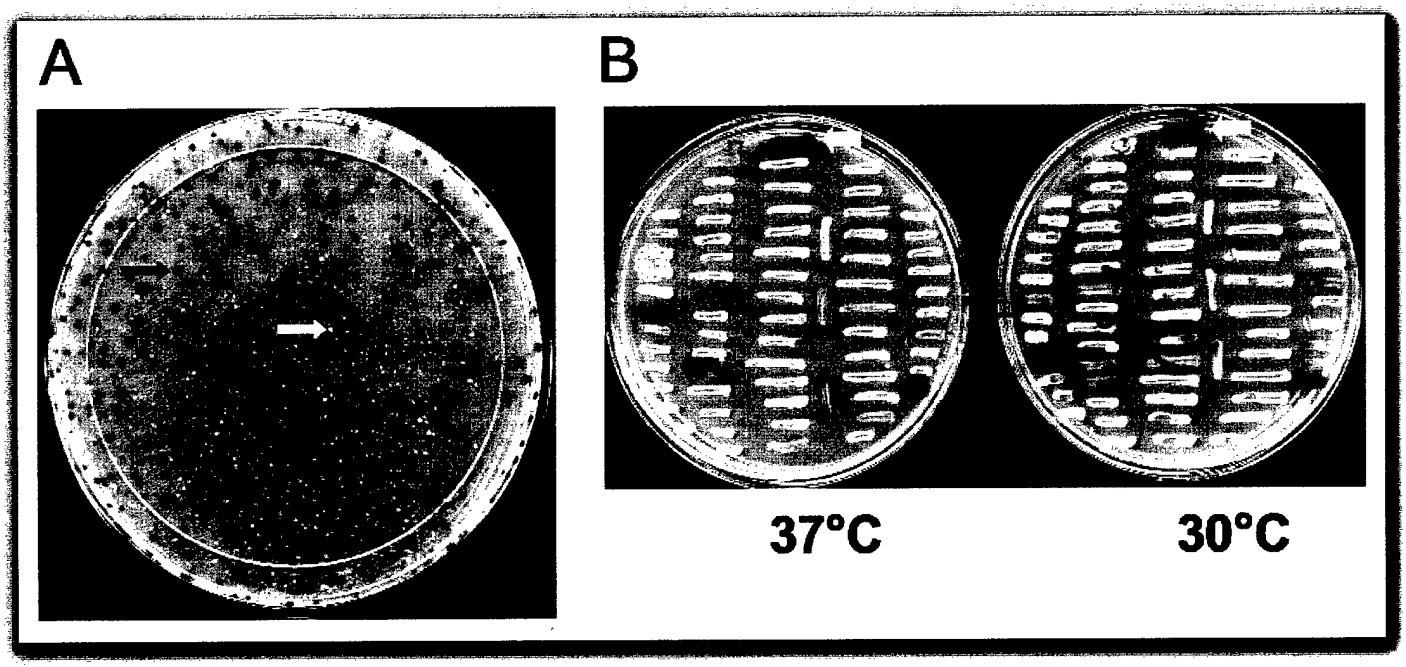

Figure 1. Screening for temperature-sensitive mutants of BRCA1 in transcription. (A) Primary screening at 37°C. Transformants carrying BRCA1 with wild-type activity appear as blue colonies (blue arrow) and transformants carrying loss-of-function mutants at 37°C appear as white colonies (white arrow). White colonies were replated in parallel and incubated at 30°C and 37°C. (B) Plates containing replicas of each white clone isolated from primary plates. A transformant carrying a wild-type BRCA1 is included at the top of each plate (white arrow). Clones that were consistently white at 37°C and blue at 30°C were isolated as temperature-sensitive mutants (blue arrow). Clones that were white at both temperatures were isolated as loss-of-function mutants.

Table 1 **TEMPERATURE-SENSITIVE MUTANTS OF BRCA1 (AA 1560–1863) IN TRANSCRIPTION**

Clone	Exon	Mutation	Nucleotide Change[a]	Allowed Residues[b]	Secondary Structure[c] and Comments	Activity[d] 30°C	Activity[d] 37°C
TS1	17	F1668S	T5122C	F	BRCT-N α-helix 1	+++	-
	24	E1836G	A5626G	DE	BRCT-C α-helix 3		
TS4	16	L1605L	T4932C	silent	unknown	+++	-
	17	V1687A	T5179C	V	BRCT-N β-sheet 3		
	19	K1727E	A5298G	KRQ	BRCT-N/BRCT-C interval		
TS6	16	L1639S	T5053C	LV	unknown	+++	-
TS19	20	F1734L	T5319C	F	BRCT-N/BRCT-C interval	+++	-
	20	E1735E	A5324G	silent	BRCT-N/BRCT-C interval		
TS25	16	S1610S	T4949C	silent	unknown	+++	-
		S1722F	C5284T	S	BRCT-N α-helix 3		
TS26	20	F1734L	T5319C	F	BRCT-N/BRCT-C interval	+++	-
TS30	20	F1734L	T5319C	F	BRCT-N/BRCT-C interval	+++	-
TS32	17	K1667E	A5118G	KR	BRCT-N α-helix 1	+++	-
		S1722F	C5284T	S	BRCT-N α-helix 3		
TS33	20	F1734L	T5319C	F	BRCT-N/BRCT-C interval	+++	-
TS36	16	S1631N	G5011A	SI	unknown	+++	-
	18	V1713A	T5257C	VI	BRCT-N β-sheet 4; uncharacterized variant found as a germline mutation[e]		
TS50	17	H1686Q	T5177A	H	BRCT-N β-sheet 3	+	-

[a]Nucleotide numbering corresponds to human *BRCA1* cDNA deposited in GenBank accession #U14680; [b]Residues that are found in the same position in an alignment of human (U14680), chimpanzee (AF207822), dog (U50709), rat (AF036760), mouse (U68174), chicken (AF355273) and frog (AF416868) homologs. [c]According to the BRCA1 BRCT crystal structure;[37] [d]Activity was scored in plates after 6 days. (+++) activity comparable to wild-type BRCA1; [e]As described in the Breast Cancer Information Core (BIC) database.

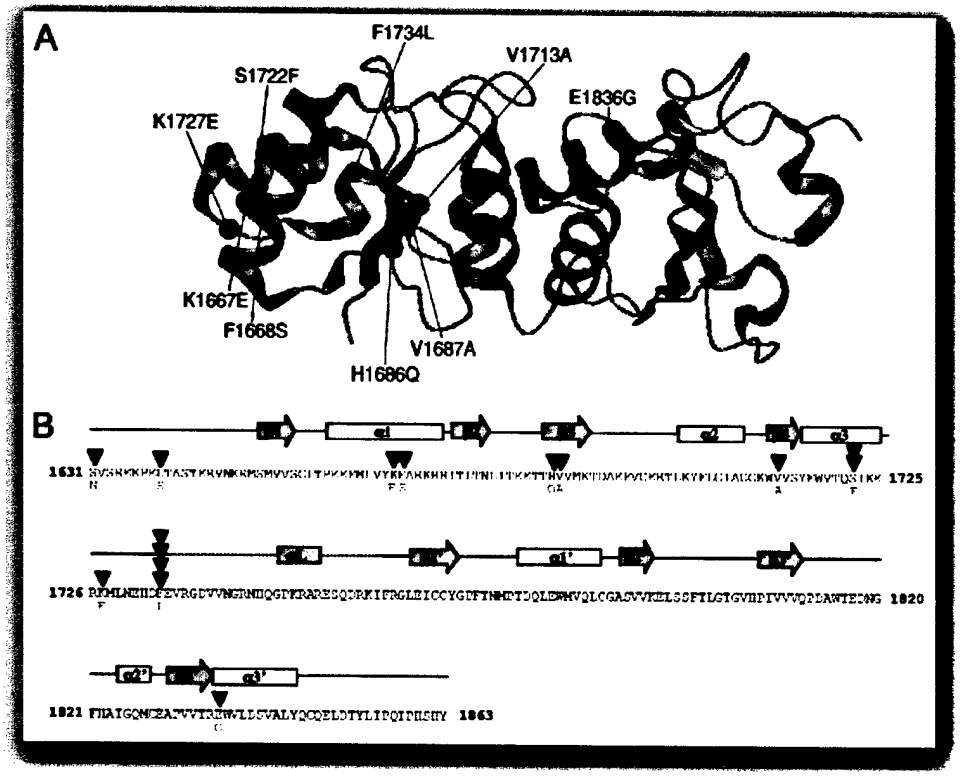

Figure 2. TS mutations localize primarily to the BRCT-N domain. (A) The location of the eight unique TS mutations is shown in the BRCT-N and BRCT-C domains of human BRCA1 according to the crystal structure of human BRCA1 BRCT region.[37] Red spheres represent the only coding change in a single clone and blue spheres represent changes that are in clones with multiple mutations. Note that with exception of S1631N, L1639S (which precede the BRCT domains and are not shown) and E1836G (TS1), all other TS mutations map to the BRCT-N domain. (B) Secondary structure elements according to crystal structure of BRCA1 BRCT region[37] are depicted above the sequence. Interval region, separating BRCT-N and BRCT-C is represented by a dotted line with a α-helix (αL; purple). Residue positions mutated in TS clones are shown for clones containing one (red triangle) or two changes (blue triangle). Changes are indicated below the sequence.

Table 2 **Loss-of-Function Mutants of BRCA1 (aa 1560-1863) in Transcription**

Clone	Exon	Mutation	Nucleotide Change[a]	Allowed Residues[b]	Secondary Structure[c] and Comments	Activity[d] 30°C	37°C
LF2	23	Q1811R	A5551G	Q	BRCT-C β3-α2 loop; uncharacterized variant found as a germline mutation[e]	-	-
	23	P1812S	C5553T	P	BRCT-C β3-α2 loop		
	24	A1843P	G5646C	AS	BRCT-C α-helix 3; uncharacterized variant found as a germline mutation[e]		
LF3	17	L1671L	A5132G	silent		-	-
	18	E1694G	A5201G	E	BRCT-N β3-α2 loop		
	24	V1842A	T5644C	VIL	BRCT-C α-helix 3		
LF5	16	L1657P	T5089C	L	BRCT-N β1-α1 loop	-	-
LF8	18	F1704S	T5230C	F	BRCT-N α-helix 2	-	-
LF15	24	A1843T	G5646A	AS	BRCT-C α-helix 3	-	-
LF20	17	T1691T	A5192G	silent	unknown	-	-
	17	F1668S	T5122C	F	BRCT-N α-helix 3		
	24	R1835R	A5624G	silent			
	24	P1856T	C5685A	PQS	unknown		
LF22	18	F1704S	T5230C	F	BRCT-N α-helix 2	-	-
LF23	18	F1704S	T5230C	F	BRCT-N α-helix 2	-	-
LF24	20	G1743R	G5346A	G	BRCT-N/BRCT-C interval	-	-
LF27	16	L1636L	T5034C	silent		-	-
	16	L1657P	T5089C	L	BRCT-N β1-α1 loop		
	17	L1664L	C5110T	silent			
LF28	16	S1577P	T4848C	S	unknown	-	-
	16	S1655P	T5082C	S	BRCT-N β1-α1 loop; residue mutated in the germline (S1655F)[e]		
LF35	21	M1775R	T5443A	M	BRCT-C β1-α1 loop; cancer-associated mutation found in the germline[e]	-	-
	22	Q1779Q	A5456G	silent			
LF34	23	I1807S	T5539G	IVL	BRCT-C β-sheet 3	-	-
	23	H1822H	T5585C	silent			
LF38	16	E1660G	G5098A	EKSC	BRCT-N α-helix 1	-	-
LF47	16	R1649R	A5066G	silent		-	-
	18	F1704S	T5230C	F	BRCT-N α-helix 2		

[a]Nucleotide numbering corresponds to human *BRCA1* cDNA deposited in GenBank accession #U14680; [b]Residues that are found in the same position in an alignment of human (U14680), chimpanzee (AF207822), dog (U50709), rat (AF036760), mouse (U68174), chicken (AF355273) and frog (AF416868) homologs. [c]According to the BRCA1 BRCT crystal structure;[36] [d]Activity was scored in plates after 6 days. (+++) activity comparable to wild-type BRCA1; [e]As described in the Breast Cancer Information Core (BIC) database.

residues in the BRCT domains. Interestingly, we recovered a recurring cancer-associated mutation of BRCA1, M1775R (LF35; Table 2).[29] Also, Q1811R and A1843P, found together in LF2, are unclassified variants listed in the Breast Cancer Information Core database (http://www.nhgri.nih.gov/Intramural_research/Lab_transfer/Bic/). Two mutations, F1704S and L1657P, were found in 4 and 2 independent clones, respectively. Mutations causing LF phenotype were found in all exons examined with the exception of exon 19.

TS Mutants in Human Cells. All unique TS clones had their activity measured in human cells using a fusion to GAL4 DBD and a luciferase reporter driven by a GAL4-responsive promoter. Negative controls used were two cancer-associated mutants, M1775R and Y1853X.[29,30] In four independent experiments, one of the mutants (TS50) reproducibly displayed significant activity at the permissive temperature. Whereas at 30°C it exhibited approximately 30% of wild-type activity, at 37°C it did not activate transcription of the reporter (Fig. 3). Western blot analysis

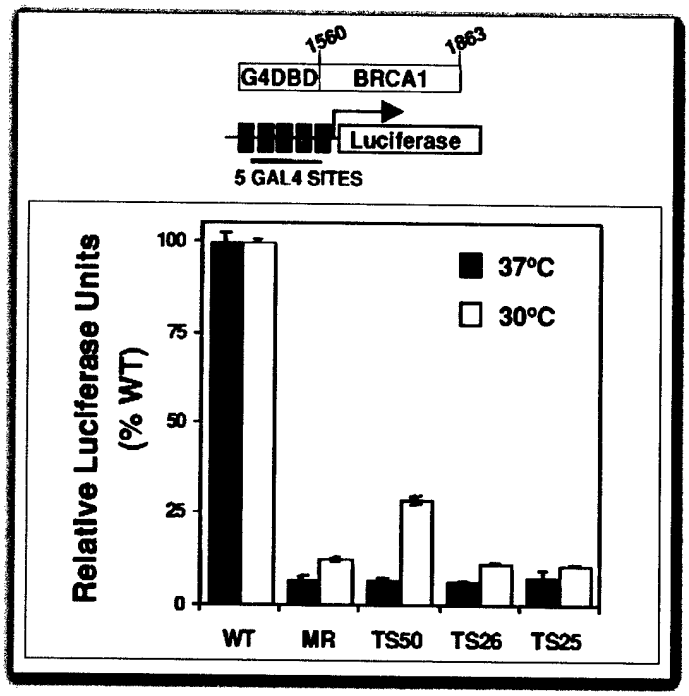

Figure 3. Transcriptional activity of TS mutants in mammalian cells. Upper panel shows a schematic representation of the GAL4-DBD:BRCA1 (aa 1560–1863) fusion protein and of the luciferase reporter gene driven by five GAL4 binding sites. Lower panel depicts the activation of luciferase expression by wild type and mutant BRCA1 constructs in 293T cells at 37°C (solid bars) or 30°C (open bars). Data were normalized to the percentage of wild-type activity at each temperature. MR, BRCA1 (aa 1560–1863) carrying the cancer-associated M1775R mutation used as negative control.

revealed that all mutant constructs were being expressed, albeit at lower levels than the WT protein (not shown).

Mammalian Two-Hybrid System. Recent reports have demonstrated that CtIP, a protein involved in transcriptional repression and a substrate of ATM, interacts with the BRCT domains of BRCA1.[28,31-34] We next examined whether the TS mutants could interact with CtIP in a temperature-dependent manner in a mammalian two-hybrid assay. We reasoned that this assay would provide a complementary approach to assess the temperature-sensitive phenotype of the mutants. Our results confirm previous reports that CtIP interacts with the carboxy-terminal region of BRCA1 and show that this interaction also occurs at 30°C (Fig. 4).[28,31] Interestingly, TS26 and TS50 were found to interact with CtIP only at 30°C. The fold induction relative to the activity of the TS mutants transfected with the VP16 transactivation domain alone (7-fold and 10-fold, respectively) was less than fold induction obtained with the WT and CtIP:VP16, suggesting that the interaction at 30°C is only partially restored. Although we observed that TS26 interacts with CtIP, it failed to activate transcription at either temperature (Fig. 3).

DISCUSSION

The function of BRCA1 has remained elusive despite extensive effort to characterize its biochemical activities. It has been implicated in DNA repair, transcription activation and repression, transcription-coupled repair, mRNA processing, cell cycle checkpoint regulation and ubiquitination.[3,4,18,35,36] We reasoned that the isolation of conditional mutants would be an important addition in the experimental armamentarium to study BRCA1. Here we developed a screening strategy to isolate mutants of the BRCA1 C-terminus that display a TS phenotype.

Our screen isolated 11 unique loss-of-function (LF) mutants (Table 2), extending our analysis of mutants that affect transcription

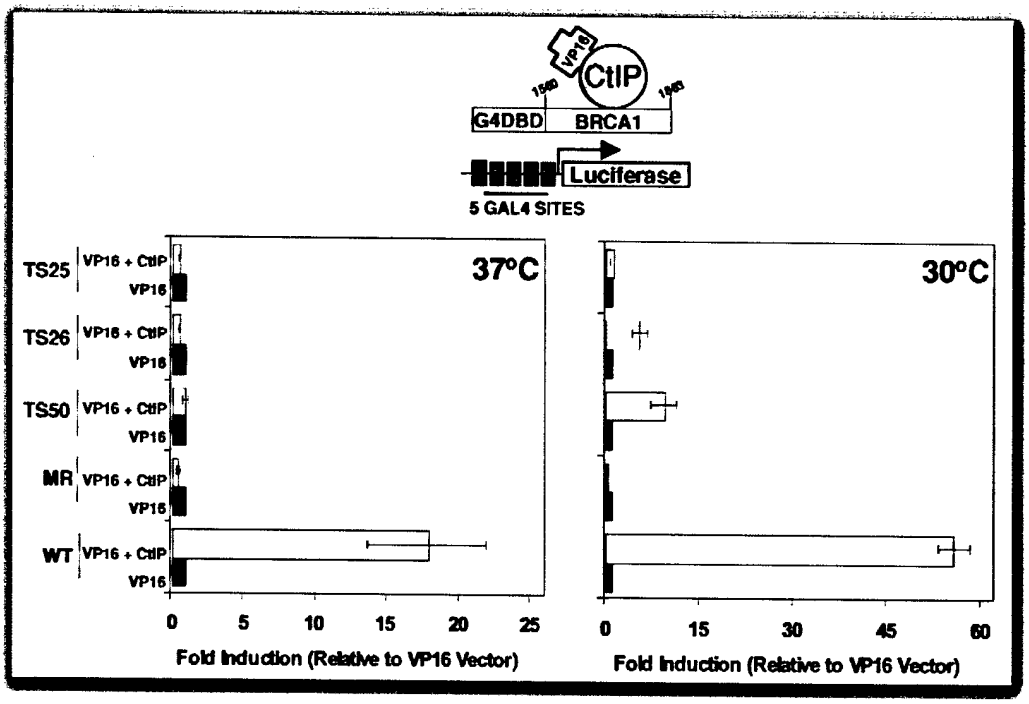

Figure 4. Mammalian two-hybrid system reveals a temperature-dependent interaction between BRCA1 TS mutants and CtIP. The upper panel shows a schematic representation of the GAL4-DBD:BRCA1 (aa 1560–1863) fusion protein used as bait, the CtIP:VP16 fusion protein used as target and the luciferase reporter gene driven by five GAL4 binding sites. The lower panel depicts the activation of the reporter gene at 37° C or 30° C by wild type and mutant BRCA1 constructs in 293T cells cotransfected with empty VP16 vector (that codes for the VP16 transactivation domain alone) or vector containing CtIP (that codes for the CtIP:VP16 fusion protein). The data were normalized to show the fold induction of transcriptional activity for each TS mutant relative to its activity when transfected with VP16 vector alone.

activation by BRCA1 and allowing us to have a more detailed picture of the structure-function features of the C-terminal region of BRCA1.[20,21] The LF mutants recovered were localized primarily in conserved hydrophobic residues at the BRCT-N and the BRCT-C domains.

We have also isolated 8 unique TS mutants using the yeast screening (Table 1, Fig. 2). One mutation, F1734L, was found in 4 independent clones in our TS set and two mutations, F1704S and L1657P, were found in 4 and 2 independent clones, respectively, in the loss-of-function set. These findings suggest that the screen might have reached saturation and therefore the mutants recovered identify important regions for the regulation of BRCA1. To understand the functional consequences of these mutations we mapped the mutations onto the crystal structure of the BRCT domain region of BRCA1 (Fig. 2A).[27] Two mutations (S1631N and L1639S) mapped to regions outside the BRCT domain and were excluded from our analysis. Significantly, all other mutations leading to temperature sensitivity, with one exception (E1836G) mapped to secondary structures in the BRCT-N and to the interval region (Fig. 2B) and cluster preferentially at the hydrophobic core of the domain (Fig. 2A). The reason for this clustering is not known but it is possible that mutations the BRCT-C have more dramatic consequences for the general folding and therefore are not stable even at lower temperatures. Alternatively, the BRCT-N may provide an important binding site to the RNA polymerase II holoenzyme, an idea that is corroborated by in vitro binding studies of BRCA1 and RNA helicase A.[9] Therefore, for mutations in the BRCT-C to affect transcription their effect has to be more dramatic allowing us to isolate only loss of function mutations.

Interestingly, mutations in residues located at hydrophobic cores in the catalytic domain of tyrosine kinases as well as in SH3 domains have been demonstrated to confer temperature-sensitivity.[38] In three clones (TS1, TS4 and TS36) two mutations were found and only one of them may be important for temperature sensitivity. Alternatively, as found in TS mutants of v-Src, two mutations may be required.[39,40]

One of the TS mutants isolated in yeast, H1686Q, displayed a temperature-dependent activation of transcription when tested in human cells (Fig. 3). This observation indicates that residue H1686 is located at a critical position for the stability of the BRCT domains (Fig. 2). In addition to the ability of TS50 to activate transcription only at the permissive temperature in mammalian cells, we found that its interaction with CtIP also occurred in a temperature-dependent manner (Fig. 4). Intriguingly, mutant TS26 interacts with CtIP at the permissive temperature but is unable to activate transcription at either 30°C or 37°C. Based on these observations we propose that TS50 can be used to clarify the physiological relevance of the BRCA1/CtIP interaction.

The inability of most of these clones to behave as TS mutants in mammalian cells may be due to inherent differences in the range of temperatures and metabolism of yeast versus the mammalian system. Alternatively, this may reflect the fact that the reporter used in the screen is not stringent. We tend to favor the latter explanation because there are documented examples of TS mutants isolated in yeast screens at 25°C and 33°C, permissive and restrictive temperature respectively, that turned out to display TS activity in mammalian cells at 34°C and 40.5°C.[38] This is a striking example in which the permissive temperature in mammalian cells was even higher than the restrictive temperature in yeast suggesting that the mutants adapt to the range of temperatures used in a particular host. The use of a low-stringency reporter is important at the restrictive temperature to guarantee the selection of mutants with the lowest possible activity. However, when screened at the permissive temperatures it will allow the selection of clones that may have low activity. We are currently exploring these different possibilities.

Although only one of the mutant clones displayed a mammalian TS phenotype in transcription, the other clones isolated here are candidates to become molecular biological tools in yeast to dissect the function of BRCA1 in transcription and to guide further efforts to isolate more relevant TS mutants in mammalian cells. If we apply a conservative interpretation of the transcriptional assay, i.e., that it is a measure of the integrity of the BRCT domain, then it is possible that the data collected here may serve as a basis to rationally design conditional mutants to other proteins that present BRCT domains in their structure. It is important to stress that the TS mutants recovered are inactive at 37°C and are likely to represent cancer-associated variants if found as germ-line mutations.

Acknowledgements

Thanks to Claudia Bernardi and José Galán for critically reading the manuscript, Nebojsa Mirkovic for the preparation of figure 2A and Richard Baer for providing the CtIP constructs.

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