THE JOURNAL OF BIOLOGICAL CHEMISTRY
© 2003 by The American Society for Biochemistry and Molecular Biology, Inc.
Vol. 278, No. 52, Issue of December 26, pp. 53007–53016, 2003
Printed in U.S.A.

# Detection of Protein Folding Defects Caused by BRCA1-BRCT Truncation and Missense Mutations* [S]

Received for publication, September 12, 2003
Published, JBC Papers in Press, October 8, 2003, DOI 10.1074/jbc.M310182200

**R. Scott Williams‡, Daniel I. Chasman§, D. Duong Hau‡, Benjamin Hui‡, Albert Y. Lau¶, and J. N. Mark Glover‡‖**

*From the ‡Department of Biochemistry, University of Alberta, Edmonton, Alberta T6G 2H7, Canada, the §Division of Preventative Medicine, Brigham and Women's Hospital, Boston, Massachusetts 02215, and ¶Variagenics/Nuvelo, Inc., Cambridge, Massachusetts 02139*

**Most cancer-associated BRCA1 mutations identified to date result in the premature translational termination of the protein, highlighting a crucial role for the C-terminal, BRCT repeat region in mediating BRCA1 tumor suppressor function. However, the molecular and genetic effects of missense mutations that map to the BRCT region remain largely unknown. Using a protease-based assay, we directly assessed the sensitivity of the folding of the BRCT domain to an extensive set of truncation and single amino acid substitutions derived from breast cancer screening programs. The protein can tolerate truncations of up to 8 amino acids, but further deletion results in drastic BRCT folding defects. This molecular phenotype can be correlated with an increased susceptibility to disease. A cross-validated computational assessment of the BRCT mutation data base suggests that as much as half of all BRCT missense mutations contribute to BRCA1 loss of function and disease through protein-destabilizing effects. The coupled use of proteolytic methods and computational predictive methods to detect mutant BRCA1 conformations at the protein level will augment the efficacy of current BRCA1 screening protocols, especially in the absence of clinical data that can be used to discriminate deleterious BRCT missense mutations from benign polymorphisms.**

Germline mutations within the breast and ovarian cancer susceptibility gene *BRCA1* predispose carriers to early-onset breast and breast-ovarian cancers (1). Accumulating evidence points to a role for the *BRCA1* protein product in the regulation of multiple nuclear functions including transcription, recombination, DNA repair, and checkpoint control (2–4). Tumor-associated mutations occur throughout the *BRCA1* coding sequence, but cluster to sequences encoding the N-terminal RING finger domain and the two carboxy-terminal repeat BRCT[1] domains (5–7).

The molecular details of how BRCA1 mutations contribute to the pathogenesis of cancer remain largely unknown. The functional significance of the BRCT region is highlighted by the high degree of sequence conservation within the BRCT regions of among mammalian, *Xenopus*, and avian BRCA1 homologues (8–10). Several lines of evidence reveal the BRCT is required for tumor suppressor function. A nonsense mutation, which removes 11 C-terminal residues of the second, BRCT (Tyr$^{1853}$ → stop), is associated with early-onset breast cancer (11). Two cancer-linked BRCT missense mutations (12) that destabilize the BRCT fold (13–15), A1708E and M1775R, ablate the double-strand break repair and transcription function of BRCA1 (16) and inhibit BRCT interactions with histone deacetylases (17), BACH1 (18), and the transcriptional co-repressor CtIP (19, 20). Furthermore, mice with homozygous targeted mutations removing the C-terminal half of BRCA1 are viable but develop tumors, suggesting the missing BRCT and/or other domains are expendable for survival, but not for tumor suppression (21).

Although all frameshift or nonsense mutations recorded in the Breast cancer Information Core (BIC) resulting in BRCA1 protein truncation are viewed as functionally deleterious (6, 7), the physiological significance of the majority of missense variants has not been determined due to the absence of a distinctive functional assay for BRCA1. More than 70 missense substitutions have been recorded that alter the primary sequence of the tandem BRCT repeats, but pedigree analysis clarifying the disease linkage of these alleles is available for only eight of these variants (6, 7, 12, 23–27). Many of these amino acid substitutions may be linked with disease but remain as unclassified in the BIC, because the presence of the allele has not been tested in the general population, or the segregation of the allele with disease within a family is unclear (6, 7).

The recent determination of the x-ray crystal structures of the rat and human BRCA1 BRCT repeat domains were important first steps toward understanding tumorigenic BRCT mutations and provide a novel platform for the interpretation of the effects of these alterations in the absence of clinical data (13, 15, 28). In the present study we directly evaluate the consequences missense mutation on the structure of the human BRCA1 BRCT repeats. Using a proteolysis-based assay to probe the BRCT for non-native conformations, we show that the majority of the tested missense and truncations alter the folding state of the BRCT. Cross-validated computational analyses using the BRCT structure and the sequences of proteins homologous to the human BRCT from other organisms further suggest that many of the unclassified BRCT missense mutations are likely to be disease-predisposing and perturb BRCA1 structure/function through protein-destabilizing effects.

* This work was supported by funding from the Canadian Breast Cancer Research Initiative, the Canadian Institutes of Health Research, the National Cancer Institute of Canada, and the Alberta Heritage Foundation for Medical Research (to J. N. M. G.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[S] The on-line version of this article (available at http://www.jbc.org) contains Supplemental Table S1.

‖ To whom correspondence should be addressed: Dept. of Biochemistry, University of Alberta, 437 Medical Sciences Bldg., Edmonton, Alberta T6G 2H7, Canada. Tel.: 780-492-2136; Fax: 780-492-0886; E-mail: mark.glover@ualberta.ca.

[1] The abbreviations used are: BRCT, BRCA1 C-terminal domain; BIC, Breast Cancer Information Core Database.

## EXPERIMENTAL PROCEDURES

### *Mutagenesis and Vector Construction*

Coding sequences for BRCT C-terminal truncations of human BRCA1 were amplified from the T7 promoter based expression vector for pLM1-BRCA1-(1646–1863) (13) using the following oligonucleotides: (fragment 1646–1859) FT7–5′-gga cga gaa ttc tta acc agg gag ctg att atg gtg aac aaa aga atg tcc atg-3′, CD6–5′-gat ctg gga tcc tca ggg gat ctg ggg tat cag-3′; (fragment 1646–1858) FT7, CD7–5′-gat ctg gga tcc tca gat ctg ggg tat cag gta-3′; (fragment 1646–1857) FT7, CD1–5′-gat ctg gga tcc tca ctg ggg tat cag gta ggt-3′; (fragment 1646–1855) FT7, CD5–5′-gat ctg gga tcc tca tat cag gta ggt gtc cag-3′; (fragment 1646–1853) FT7, CD4–5′-gat ctg gga tcc tca gta ggt gtc cag ctc ctg-3′; (fragment 1646–1852) FT7, 1853Ystop-5′-gat ctg gga tcc tca ggt gtc cag ctc ctg gca-3′; (fragment 1646–1851) FT7, CD3–5′-gat ctg gga tcc tca gtc cag ctc ctg gca ctg-3′; (fragment 1646–1849) FT7, CD2–5′-gat ctg gga tcc tca ctc ctg gca ctg gta gag-3′; (fragment 1646–1829) FT7, 1829stop-5′-gat ctg gga tcc tca aca cat ctg ccc aat tgc-3′; (fragment 1646–1805) FT7, 1805stop-5′-gat ctg gga tcc tca gac acc tgt gcc aag ggt-3′. The 5′ primer FT7 incorporates a ribosome binding site and an EcoRI site for cloning. The 3′ oligonucleotides include the relevant stop codons and a BamHI restriction site. Gel-purified PCR products were digested with EcoRI and BamHI and cloned into BamHI-EcoRI-digested pLM1 (29).

All BRCT single amino acid substitutions were introduced into the BRCT fragment 1646–1859. For missense mutations A1708E, M1775R, and W1837R, mutated BRCA1 coding sequences were used as template for PCR amplification with the FT7 and CD6 primers. All other missense substitutions were engineered using PCR splicing methods (30). Primary PCR mutagenesis reactions used oligonucleotide FT7 with the appropriate reverse (R) mutagenesis oligonucleotide (see below) and CD6 with the appropriate foward (F) mutagenesis oligonucleotide. PCR products from the primary reactions were gel-purified from 1.5% agarose gels using a QIAEX2 kit (Qiagen) and mixed together with oligonucleotides FT7 and CD6 in the secondary PCR splicing reactions to generate mutated PCR products that were subsequently digested with EcoRI/BamHI and ligated to pLM1. The mutagenesis oligonucleotides used were: D1692Y, F-5′-gtt atg aaa aca tat gct gag ttt gtg-3′, R-5′-cac aaa ctc agc ata tgt ttt cat aac-3′; F1695L, F-5′-aca gat gct gag ctt gtg tgt gaa cgg-3′, R-5′-ccg ttc aca cac aag ctc agc atc tgt-3′; V1696L, F-5′-gat gct gag ttt ttg tgt gaa cgg aca-3′, R-5′-tgt ccg ttc aca caa aaa ctc agc atc-3′; C1697R, F-5′-gct gag ttt gtg cgt gaa cgg aca ctg-3′, R-5′-cag tgt ccg ttc acg cac aaa ctc agc-3′; R1699W, F-5′-ttt gtg tgt gaa tgg aca ctg aaa tat-3′, R-5′-ata ttt cag tgt cca ttc aca cac aaa-3′; R1699Q, F-5′-ttt gtg tgt gaa cag aca ctg aaa tat-3′, R-5′-ata ttt cag tgt ctg ttc aca cac aaa-3′; S1715R, F-5′-aaa tgg gta gtt aga tat ttc tgg gtg-3′, R-5′-cac cca gaa ata tct aac tac cca ttt-3′; W1718C, F-5′-gtt agc tat ttc tgt gtg acc cag tct-3′, R-5′-aga ctg ggt cac aca gaa ata gct aac-3′; T1720A, F-5′-tat ttc tgg gtg gcc cag tct att aaa-3′, R-5′-ttt aat aga ctg ggc cac cca gaa ata-3′; G1738E, F-5′-ttt gaa gtc aga gaa gat gtg gtc aat g-3′, R-5′-cat tga cca cat ctt ctc tga ctt caa a-3′; G1738R F-5′-ttt gaa gtc aga aga gat gtg gtc aat g-3′, R-5′-cat tga cca cat ctc ttc tga ctt caa a-3′; P1749R, F-5′-aac cac caa ggt cgt aag cga gca aga g-3′, R-5′-ctc ttg ctc gct tac gac ctt ggt ggt t-3′; R1751Q, F-5′-caa ggt cca aag caa gca aga gaa tcc-3′, R-5′-gga ttc tct tgc ttg ctt tgg acc ttg-3′; A1752P, F-5′-ggt cca aag cga cca aga gaa tcc cag-3′, R-5′-ctg gga ttc tct tgg tcg ctt tgg acc-3′; I1766S, F-5′-agg ggg cta gaa agc tgt tgc tat ggg-3′, R-5′-ccc ata gca aca gct ttc tag ccc cct-3′; M1783T, F-5′-caa ctg gaa tgg acc gta cag ctg tgt g-3′, R-5′-cac aca gct gta cgg tcc att cca ggt t; G1788V, F-5′-gta cag ctg tgt gtt gct tct gtg gtg-3′, R-5′-cac cac aga agc aac aca cag ctg tac-3′; V1804D, F-5′-ctt ggc aca ggt gac cac cca att gtg-3′, R-5′-cac aat tgg gtg gtc acc tgt gcc aag-3′; V1809F, F-5′-cac cca att gtg ttt gtg cag cca gat-3′, R-5′-atc tgg ctg cac aaa cac aat tgg gtg-3′; W1837G, F-5′-gtg acc cga gag ggg gtg ttg gac agt g-3′, R-5′-cac tgt cca aca ccc cct ctc ggg tca c-3′. All vectors were sequenced to confirm the success of the mutagenesis reactions.

### *Proteolysis Assays*

0.2–0.5 μg of pLM1 plasmid encoding the BRCT variants were used directly as template for protein synthesis reactions with the TNT-Quick *in vitro* transcription/translation system (Promega). Immediately prior to proteolytic digestion, proteins were translated and labeled with [$^{35}$S]methionine at 30 °C for 2 h. The reticulocyte lysates were then centrifuged for 2 min at 10,000 × *g* to remove insoluble material, and 3 μl of the lysate supernatants containing the labeled translation products were added to 12 μl of digestion buffer (150 mM NaCl, 50 mM potassium phosphate, pH 7.5) containing increasing concentrations of trypsin (Sigma) or 1-chloro-3-tosylamido-7-amino-2-heptanone or $N^{\alpha}$-*p*-tosyl-L-lysine chloromethyl ketone-treated chymotrypsin (Sigma). After digestion at 20 °C for 12 min, the reactions were stopped with phenylmethylsulfonyl fluoride. Digestion products were electrophoresed on 15% SDS-PAGE gels and visualized with a phosphorimaging plate and a Molecular Dynamics Typhoon scanner. A local average background correction was used during quantification of the reaction products with ImageQuaNT (Amersham Biosciences).

### *Molecular Graphics*

Structural diagrams were created with Bobscript (31, 32) and rendered using Povray (www.povray.org).

### *Computational Analysis of Risks Associated with Missense Mutations*

*Method 1: Structure and Sequence-based Analysis*—A probability of an effect on function for the missense mutations in BRCT was determined exactly as described using both feature set A and feature set B in Ref. 33. Briefly, the crystal structure (PDB ID: 1JNX), the multiple sequence alignment for proteins homologous to human BRCT (see Fig. 3), and the chemical nature of the amino acid substitution are used to compute the values of features that are useful for predicting the effects of amino acid substitutions on protein function. For example, the quantitative estimate of solvent accessibility for a residue in a structure or its normalized phylogenetic entropy from a multiple sequence alignment are both features that can be viewed as having a quantitative relationship to the probability of an effect on function for the introduction of a mutant amino acid (33). A probability of an effect on function for a test mutation is estimated by conditional probability as the fraction of training mutations derived from exhaustive mutagenesis of the Lac repressor (34) and T4 lysozyme (35) with an effect on function from among those with feature values are similar to the feature values of the test mutation.

*Method 2: Refined Sequence-based Analysis*—The sequence-based procedure[2] for predicting the functional consequences of a mutation in a residue of human BRCT is an extension of the direct inspection of alternative amino acid existing at the corresponding residue in proteins homologous to the human BRCT. In essence, mutations that introduce amino acids observed at the corresponding residue of homologous proteins are judged to be tolerated by the human BRCT. The extension involves inferring, through the use of the Blocks9 mixture of Dirichlet priors (36), the more likely of two hypotheses explaining why some amino acids are not observed at corresponding residues of homologous proteins. Either the sequences of the homologous proteins represent an incomplete sampling of all 20 amino acids at a mutated residue position or some of the 20 amino acids are incompatible with the structural and functional constraints on the mutated residue position. Inclusion of the alternative amino acid from a mutation in the inferred set of acceptable amino acids is evidence for its compatibility with biological function. Exclusion leads to a prediction of incompatibility.

## RESULTS

### *Structural Effects of BRCT Truncation Mutations*

We previously demonstrated that the tandem BRCT repeat region of human BRCA1 forms a proteolytically resistant globular domain and that a cancer-linked mutation, Y1853ter, which removes the 11 C-terminal residues of the protein, reduces this proteolytic stability (13). To determine to what extent the BRCT fold could tolerate truncation mutations, we subjected a series BRCT deletion mutants to a proteolytic sensitivity assay (Fig. 1, see “Experimental Procedures”). The oncogenic mutation Y1853ter and all larger C-terminal deletions of the protein were degraded by the lowest concentrations of trypsin, whereas the full-length BRCT (aa 1646–1863) is highly resistant to cleavage (Fig. 1). Included with these mutations are the truncation protein products of two of the most common BIC frameshift mutants, 5382insC and IVS21–36del510, that result in stop codons at positions 1829 and 1805 of the BRCA1 coding sequence (7) (Figs. 2 and 3). Thus, BRCT folding defects resulting from cancer-predisposing BRCA1 truncation mutations can be assayed for and detected at the protein level using a simple protease sensitivity assay.

The deletion experiment also demonstrates the protein can tolerate removal of up to 8 residues, but further deletion from

[2] A. Y. Lau and D. I. Chasman, submitted for publication.

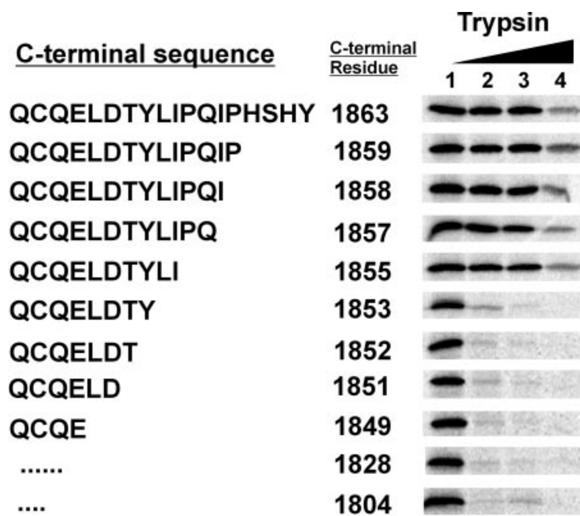



FIG. 1. **Destabilization of the BRCT domains by truncation mutation.** The indicated BRCT truncation mutations were *in vitro* transcribed and translated, and then digested with increasing amounts of trypsin. *Lanes 1–4*: 0, 6, 60, and 600 μg/ml trypsin. Translated protein products with C termini at positions 1804, 1828, and 1852, correspond to the deletion products of the cancer-predisposing mutations whose truncation effects are depicted in Fig. 2.

the C terminus greatly impairs the native folding of the domain, rendering it highly sensitive to proteolysis (Figs. 1 and 2*D*). Consistent with this finding, the transcriptional activation activity of the BRCT domains was abolished by C-terminal deletions that truncate beyond a hydrophobic pair of residues, $Leu^{1854}$ and $Ile^{1855}$ (24). These hydrophobes mark the C-terminal boundary for conservation of mammalian, avian, and *Xenopus* BRCA1 homologues (Fig. 3) and make critical aliphatic contacts to the β-sheet of the C-terminal BRCT in the structures of the human and rat BRCA1-BRCT repeats (Fig. 2*D*) (13, 28). Hence, the transcriptional activation defects observed for BRCT deletion mutants likely result from destabilization of the protein.

### *Missense Substitutions Destabilize the BRCT*

Similar to the truncation mutants, two cancer predisposing missense mutations, A1708E and M1775R, are destabilizing and exhibit altered BRCT protease susceptibility (13–15). To gain insights into the effects of other patient-derived mutations recorded in the BIC, we generated 23 additional missense variants and tested these proteins for proteolytic sensitivity (see "Experimental Procedures," Fig. 4). 20/25 of the missense mutations tested showed varying degrees of enhanced sensitivity to tryptic digestion at 20 °C (Fig. 4*A*). Five of six of the mutations that substitute an arginine into the protein (C1697R, S1715R, G1738R, P1749R, and W1837R) also show increased sensitivity to chymotryptic cleavage at 20 °C (Fig. 4*B*) suggesting that destabilizing effects, rather than the introduction of a new trypsin cleavage site, are responsible for the protease sensitivity. Mutant M1775R is also clearly destabilizing (14) and shows sensitivity to chymotrypsin at elevated temperatures (15).

The expression levels of the BRCT variants in the reticulocyte lysates typically range between 0.3- and 1.2-fold of wild type levels. Because the expressed variants constitute less than 5–10% of the total protein digested in the lysates and we are using logarithmic increases in trypsin concentrations, we can quantify the percentages of protein remaining following digestion at each level of protease and directly compare these values to establish a proteolysis-based hierarchy for the severity of the destabilizing effects (Fig. 5). Here we define highly destabiliz-

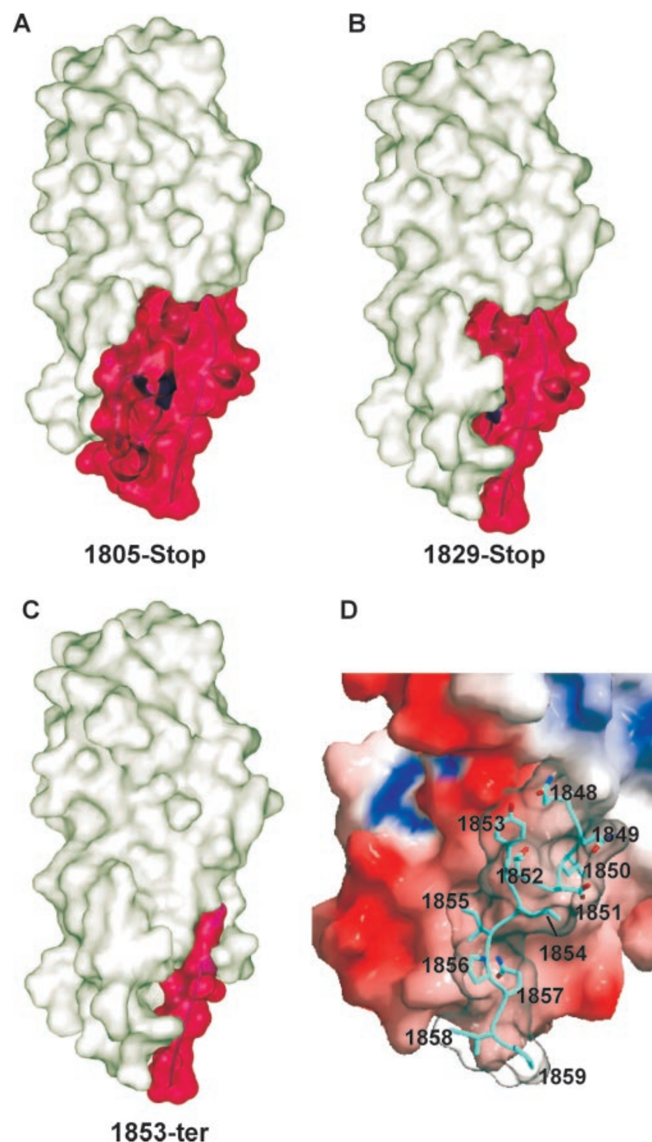



FIG. 2. **Structural effects of cancer-associated BRCA1 BRCT truncation-causing mutations.** *A*, a stop codon at position 1805 results from frameshift IVS21–36del510, removing much of the C-terminal BRCT domain. *B*, frameshift 5382insC creates a stop codon at position 1829 in BRCA1 and is one of the most commonly recorded BIC mutations. *C*, a nonsense mutation 1853-ter results in the removal of the 11 C-terminal residues of the protein and is linked to disease. For *A–C*, *red portions* of the structures are deleted residues caused by truncation mutations. *D*, interaction of the C-terminal tail of BRCA1 with BRCT-C. Negative electrostatic potential is *red* and positive is *blue*. The C terminus of BRCA1 forms a $3_{10}$ helix and an extended peptide that packs against α2′ and the β-sheet. C-terminal deletions beyond the hydrophobic residues $Leu^{1854}$ and $Ile^{1855}$ are destabilizing.

ing mutations as those mutants for which >60% of the protein is degraded at the lowest concentration (6 μg/ml) of trypsin. Intermediately destabilizing variants are >60% degraded at the intermediate trypsin concentration (60 μg/ml). Finally, the mutants showing wild type digestion profiles, with limited degradation until exposure to the highest trypsin concentration, are classified as having no destabilizing effect. Based on these criteria, the majority of the variants (13/25) are highly destabilizing, 7/25 are intermediately destabilizing, and 5/25 have no apparent effect.

Homology models of the human BRCA1-BRCT repeats, built from the XRCC1 C-terminal BRCT structure (37), have been used to describe structural environments of BRCA1-BRCT missense variants (38). Because these descriptions are inaccurate

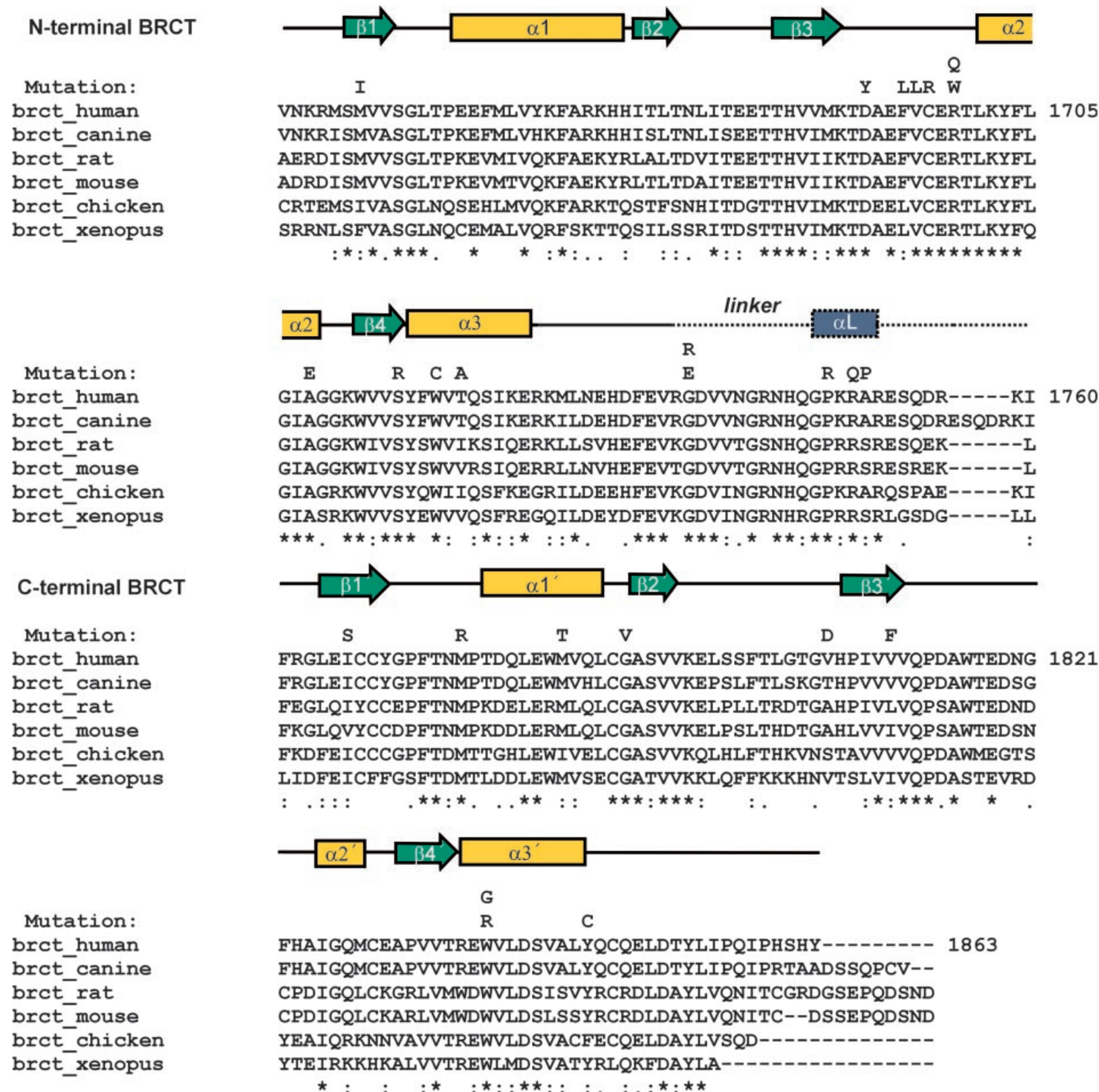



FIG. 3. **Amino acid sequence alignment of the BRCT repeat region of cloned BRCA1 homologues.** Secondary structure elements are from the human BRCT repeat structure, RCSB: 1JNX. The positions of the 25 missense mutations studied here are indicated. Numbering is for Human BRCA1. Alignments were created with ClustalX (NCBI accession numbers: human, AAA73985; canine, AAD56289; rat, AAC36493; mouse, AAD00168; chicken, AAK83825; *Xenopus*, AAL13037).

in many respects, we have reclassified the BRCT missense mutations into the four following categories based on their distribution in the human BRCT repeat structure (13) (Fig. 5 and Table I) as follows.

*Surface Mutations*—This class of mutations includes amino acid residues found on the surface of the BRCT that appear to make little contribution to the structure of the domain. Three of five of the tested surface mutants (F1695L, T1720A, and V1804D) have no destabilizing effect. Two exposed mutations localized to the $\beta$3-$\alpha$2 connecting loop (D1692Y and V1696L) confer moderate protease susceptibility to the domain (Fig. 5*A*). As highlighted by Joo *et al.* (28), this loop forms a extended $\beta$-hairpin structure, participates in the formation of one of the two conserved BRCT surfaces targeted by missense mutations, and may be the primary site of interaction with the BRCA1-associated helicase BACH1. Hence, the BACH1 binding defect reported for mutation F1695L likely results from disruption of a contact site, whereas reduced BACH1 binding for the V1696L mutation may be due to a destabilizing effect, disruption of the contact site, or both.

*BRCT Interface Mutations*—The BRCA1 C-terminal domain consists of two BRCT repeats that pack together head-to-tail via a conserved triple-helical interface, and several of the key residues mediating these BRCT-BRCT contacts are targeted by mutation (Fig. 5*B* and Table I) (13). Four of five of the tested BRCT interface mutants are destabilizing. Three destabilizing mutants, A1708E, M1775R, and M1783T, likely disrupt the hydrophobic packing between the repeats. The crystal structure of the M1775R variant revealed that mutation-induced structural rearrangements, including flipping of the mutated arginine out of the hydrophobic core of the protein, contribute to fold destabilization (15). The intermediate protease sensitivity of mutation M1783T likely results from combined deleterious effects of protein core cavitation and the burial of a polar hydroxyl group at the BRCT interface. Residue Arg[1699] normally participates in a salt bridge between the BRCT repeats. The loss of salt-bridging

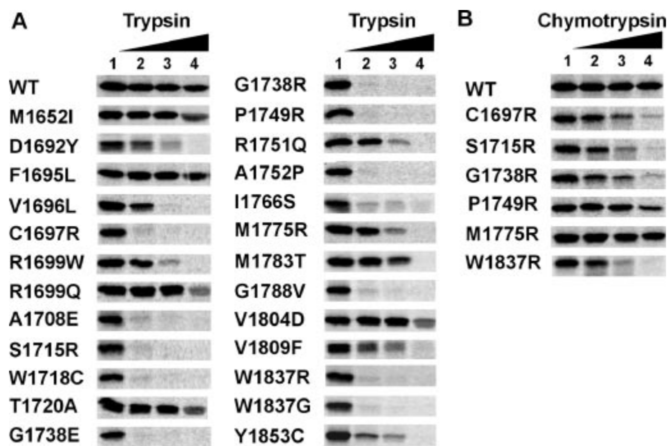



FIG. 4. **Destabilization of the BRCT domains by missense mutations.** *A*, the indicated missense mutations were digested with increasing concentration amounts of trypsin. *Lanes 1–4*: 0, 6, 60, and 600 μg/ml trypsin. *B*, mutations harboring a trypsin cleavage site were digested with chymotrypsin. *Lanes 1–4*: 0, 6, 60, and 600 μg/ml chymotrypsin.

interactions and steric strain associated with accommodating the tryptophan may contribute to conformational instability of the R1699W mutant. The intermediate stability of R1699W explains the temperature-sensitive transcription phenotype ascribed to this mutation (27). Conversely, R1699Q has little to no effect on BRCT structure and appears to have little effect on transcription activation (25).

*BRCA1 Fold Mutations*—We have designated a third class of mutations as BRCA1 fold mutants. These substitutions include residues that participate in folding of the BRCT linker region, and residues that do not fall at BRCT fold special positions, but are buried and conserved among BRCA1 homologues (Fig. 5*C*). All of the tested BRCA1 fold class mutants tested alter the folding of the domain. The majority of these mutants (C1697R, S1715R, G1738R, G1738E, and P1749R) introduce charged residues into the protein core and are highly destabilizing. The A1752P mutant likely disrupts the linker helix and is highly destabilizing. The position of $Arg^{1751}$ in the crystal structure of human BRCA1 is unclear, but the equivalent residue in the rat structure indicates this residue is involved in salt bridging interactions and the packing of BRCT linker helix (28). This arginine is conserved among all known BRCA1 homologues, and the R1751Q mutation may disrupt similar electrostatic stabilization in the human protein.

*BRCT Fold Mutations*—A conserved hydrophobic clustering signature for the BRCT fold superfamily of proteins was originally identified using sequence-based methods (39, 40). Residues at these positions dictate the fold of an individual BRCT and participate in formation of the BRCT hydrophobic core or are found in turns. As shown in Fig. 5*D*, 7 of 8 of the mutations tested (W1718C, I1766S, G1788V, V1809F, W1837G, W1837R, and Y1853C) at BRCT fold positions are intermediately or highly destabilizing. The highly destabilizing mutations W1718C, W1837G, and W1837R mutate the invariant BRCT fold tryptophan of both the N- and C-terminal BRCT domains. This critical core residue appears intolerant to both cavitating (W1718C and W1837G) or charge substitution mutations (W1837R) and mediates van der Walls contacts from helix $\alpha 3$ to other secondary structure elements of the BRCT fold, including $\beta$-sheet, helix $\alpha 1$, and the $3_{10}$ helix. Mutation G1788V disrupts the conserved tight turn between $\alpha 1'$ and $\beta 2'$ of the C-terminal BRCT. Figs. 2*D* and 5*D* highlight the role of Y1853 in positioning the C-terminal BRCT $3_{10}$ helix that packs against the $\beta$-sheet, and substitution of this residue with a cysteine is highly destabilizing. Two of the three tested BRCT fold class mutants that target residues that contribute to intra $\beta$-sheet packing, I1766S and V1809F, are destabilizing. The third $\beta$-sheet mutant, M1652I, has been classified as a benign polymorphism (41, 42) and does not increase the protease sensitivity of the domain.

### *Mutations That Destabilize the BRCT Predispose Carriers to Disease*

Pedigree analysis clarifying the disease predisposition of *BRCA1* alleles is currently available for 8 of the 79 reported BRCA1-BRCT single amino acid substitution variants (Table I). Seven of the mutations, D1692Y, C1697R, R1699W, A1708E, S1715R, P1749R, and M1775R, are destabilizing (Figs. 4 and 5) and are linked to cancer (Table I). In contrast, the frequently recorded BIC polymorphism M1652I exhibits no structural defect, indicating the protease stability assay can successfully discriminate benign mutations from disease-causing variants. The cancer-associated truncation mutants are also protease sensitive (Fig. 2).

Taken together, these results indicate that protease-based detection of altered BRCT stability provides a novel and powerful predictive tool that can be used to assess disease linkage of BRCT mutations in instances where pedigree data is not available. Thus, we suggest that the 20 destabilizing missense mutants and truncations greater than eight amino acids are cancer-predisposing.

### *Predicting the Structural Consequences of Mutation on the BRCT*

The recent development of computational methods that incorporate detailed structural and sequence information to predict the effects of single amino acid substitutions on protein structure/function provides us with alternative tools to study the BRCT mutations (33).[2] We have applied two independent methods to predict the potential effects of the 25 missense mutations studied here (Table I) and all known BRCT single amino acid substitutions recorded in the breast cancer information core (www.nhgri.nih.gov/Intramural_research/Lab_transfer/Bic/) (Supplementary Table I).

In the first computational method, a set of quantitative and qualitative features are defined for each amino acid substitution in the BRCT based on its structural disposition, an assessment of the sequence conservation mapped onto the structure, and the potential consequences of introducing the alternative amino acid into the structural environment of the mutated residue. An overall probability of an effect on function is then calculated by comparing the values of the features to the values of features of a large number of mutations with known effects on function (in this case from the Lac repressor and T4 lysozyme (33)). Predictions using two sets of features (A and B in Ref. 33) yielded similar results (see Supplementary Table I). About 51% (35 out of 69) of the mutations are predicted to effect BRCT structure or function with a probability greater than 0.5. Comparison to the experimental analysis of the mutations by proteolysis indicated disagreement for six of 22 of the mutations (0.27), consistent with previous estimates of the cross-validated error described previously (Table I) (33).

The second computational methodology estimates potential effects of mutations on function by extracting as much information as possible from the observed amino acid substitutions in the comparison of the human BRCT sequence to its homologues from other organisms. The method[2] relies extensively on Bayesian prior information representing empirically observed amino acid exchangeability in a large number of sequence families (BLOCKS data base (36) and Blocks9 priors (43)) to infer whether an alternative amino acid introduced by muta-

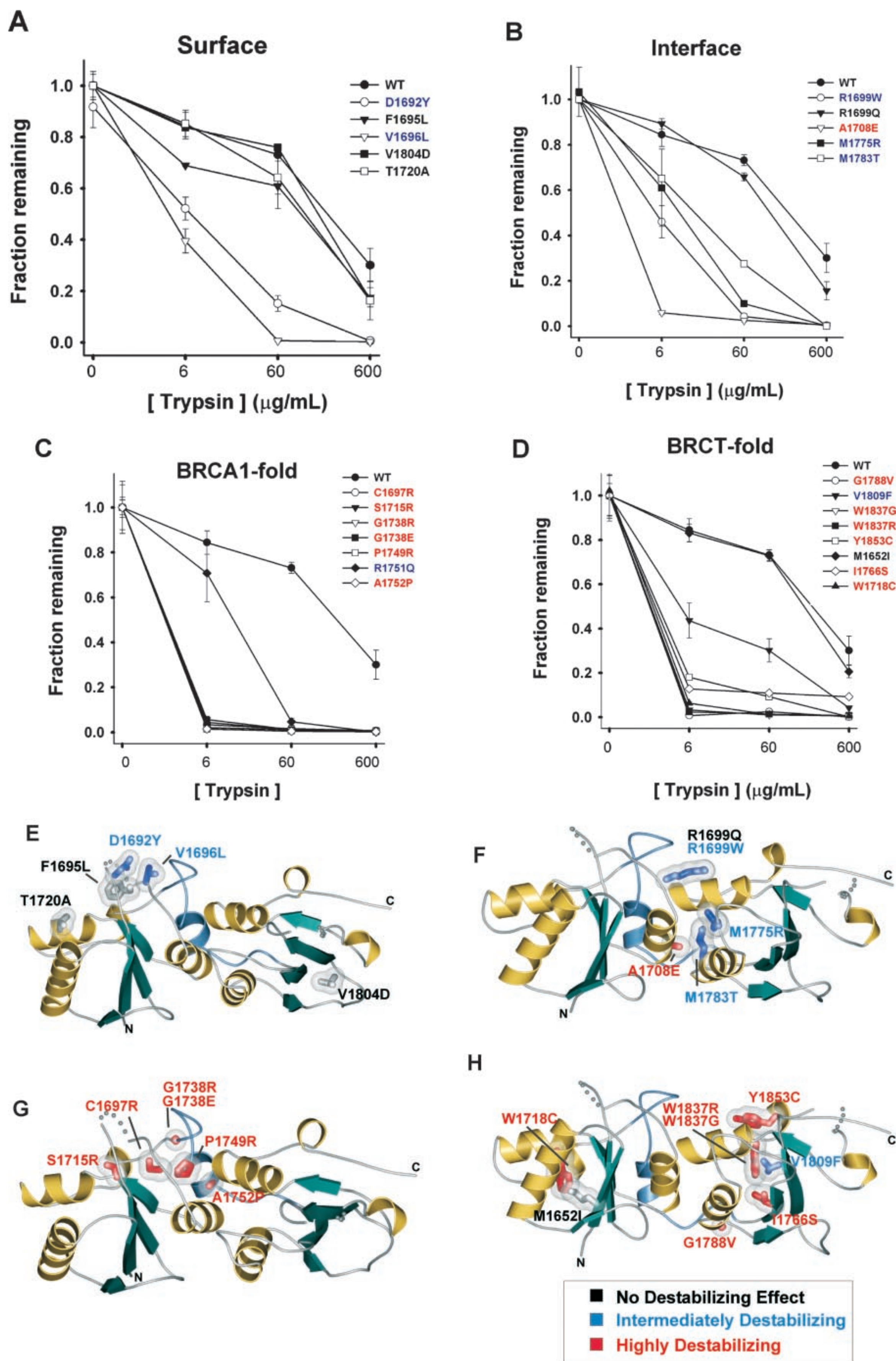



FIG. 5. **Quantification and classification of the structural effects of BRCT missense mutations.** *A–D*, the tested BRCT missense mutations have been divided into four classes based on their distribution in the BRCT structure (see text). The fraction remaining is the percentage of starting protein present following digestion with the indicated concentrations of trypsin. Data points are the mean value of digestions performed

TABLE I
*Structure, function, and disease effects of BRCT missense mutations*

| Mutant | Secondary structure[a] | Mutant class | Protease sensitivity[b] | Predictive method 1[c] | Predictive method 2[d] | Transcription[e] | Disease effects[f] | Solubility and stability[g] |
|---|---|---|---|---|---|---|---|---|
| M1652I | β | BRCT fold | (−) | (+) | (−) | t/c(−)[h] | (−) | Soluble |
| D1692Y | c | Surface | (+) | (−) | (+) | | (+) | Insoluble |
| F1695L | c | Surface | (−) | (−) | (−) | | ? | Soluble |
| V1696L | c | Surface | (+) | (−) | (−) | | ? | Soluble |
| C1697R | c | BRCA1 fold | (++) | (+) | (+) | t/c (+) | (+) | Insoluble |
| R1699W | c | Interface | (+) | (+) | (+) | t/c (−) | (+) | Soluble |
| R1699Q | c | Interface | (−) | (+) | (−) | t/c (−) | ? | Soluble |
| A1708E | α | Interface | (++) | (+) | (+) | t/c (+) | (+) | Insoluble |
| S1715R | β | BRCA1 fold | (++) | (+) | (+) | t/c (+) | (+) | |
| W1718C | α | BRCT fold | (++) | (+) | (+) | | ? | |
| T1720A | α | Surface | (−) | (−) | (−) | | ? | |
| G1738E | c | BRCA1 fold | (++) | (+) | (+) | t/c (+) | (+) | Insoluble |
| G1738R | c | BRCA1 fold | (++) | (+) | (+) | | ? | Insoluble |
| P1749R | α | BRCA1 fold | (++) | (+) | (+) | | ? | Insoluble |
| R1751Q | α | BRCA1 fold | (+) | (−) | (−) | | ? | Soluble |
| A1752P | α | BRCA1 fold | (++) | (−) | (−) | | ? | |
| I1766S | β | BRCT fold | (++) | (+) | (+) | | ? | Insoluble |
| M1775R | c | Interface | (+) | (−) | (+) | t/c (+) | (+) | $\Delta\Delta G$ = 5.0 kcal/mol |
| M1783T | α | Interface | (+) | (+) | (−) | | ? | $\Delta\Delta G$ = 4.28 kcal/mol |
| G1788V | c | BRCT fold | (++) | n/a | (+) | | ? | Insoluble |
| V1804D | c | Surface | (−) | (−) | (−) | | ? | |
| V1809F | β | BRCT fold | (+) | (−) | (−) | | ? | Soluble |
| W1837R | α | BRCT fold | (++) | (+) | (+) | | ? | Insoluble |
| W1837G | α | BRCT fold | (++) | (+) | (−) | | ? | Insoluble |
| Y1853C | α | BRCT fold | (++) | (+) | (+) | | ? | Soluble |

[a] Secondary structure is from the human BRCT domain structure (13).
[b] Protease sensitivity: (−), wild type, no effect; (+), intermediately destabilizing; and (++), highly destabilizing.
[c] Predictive method 1. Predicted effect on function is as described by Chasman and Adams (33). (+): The mutation is predicted to effect structure/function, the probability of an effect on function is >0.5. (−): The mutation is predicted to be a benign substitution, the probability of an effect on structure/function is <0.5 (see Supplementary Table I for calculated probabilities). For one mutation, G1788V, there were too few data points to estimate a probability.
[d] Predictive method 2, sequence based. (+), The mutation is predicted to effect structure/function; (−), The mutation is predicted to be a benign substitution.
[e] Transcription effects are those reported by Monteiro *et al.* (45), Monteiro *et al.* (42), Hayes *et al.* (24), Vallon-Christersson *et al.* (25), and Worley *et al.* (27).
[f] Disease linkage data are from recorded entries in the BIC (Hayes *et al.* (24), Vallon-Christersson *et al.* (25), and T. S. Frank, personal communication). (+), linked to disease; (−), not linked; (?), unknown.
[g] Stabilities reported from Ekblad *et al.* (14). The reported solubility in *E. coli* is from this study or Ekblad *et al.* (14).
[h] t/c (−), no effect on transcription; t/c (+), affects t/c.

tion is consistent with the biological and chemical character of amino acids found at the corresponding residues of BRCT homologues. This method predicts 14 of 25 of the mutations tested by proteolysis are incompatible with normal BRCT function, representing agreement with the experimental data for 19 of them (0.76). The first method combining structure and sequence is consistent with predictions from method 2 for 18/24 mutations (0.75), and both methods agree with the proteolysis data for 14/24 (0.58) of the mutations.

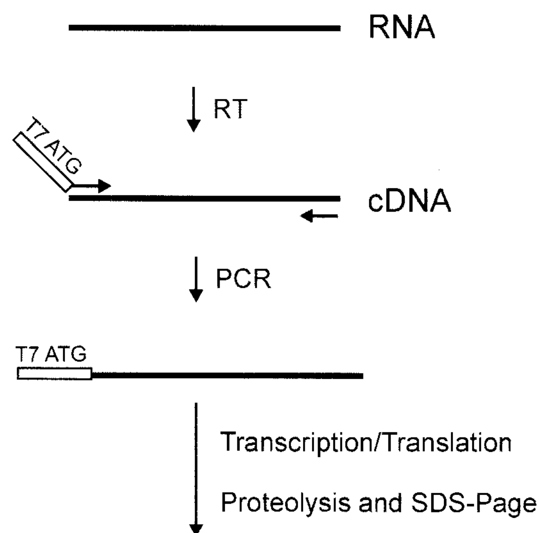
## DISCUSSION

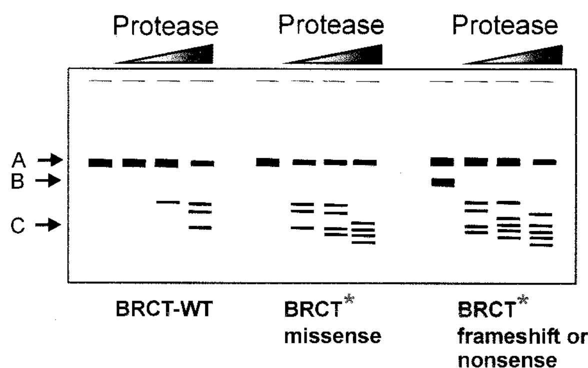
*Protein Destabilization Ablates BRCT-mediated Transcriptional Activation*—When tethered to a GAL4 DNA binding domain, the BRCT domains can activate transcription in yeast and mammalian systems (44–46). Significantly, potential targets of BRCA1 transcriptional regulation include the p53-responsive genes encoding p21 as well as GADD45 (47, 48) suggesting that BRCA1 has a role in regulating DNA repair and checkpoint controls. The BRCT may modulate these functions through direct recruitment of the RNA polymerase holoenzyme (49, 50); however, the physiological significance of these effects and the precise biochemical mechanism by which the BRCT activates transcription remains unclear (reviewed in Ref. 2). Nevertheless, this intrinsic activity forms the basis for a BRCA1 functional assay that has been used to probe for defects caused by several BRCT missense mutations (24, 25, 27).

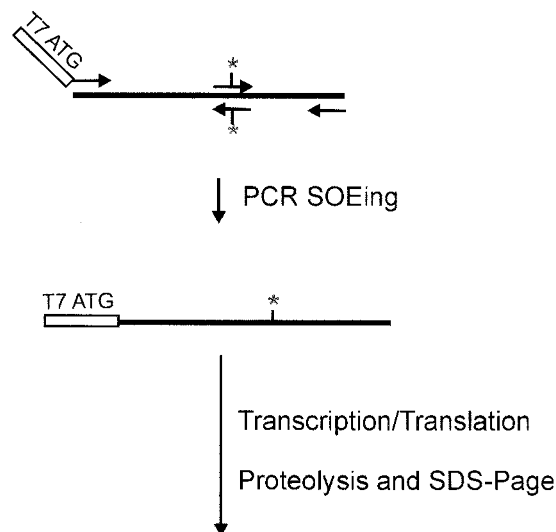
Comparison of the transcription and protease-based assay data reveal a striking correlation between destabilizing phenotypes and transcriptional defects (Table I). That is, less stable BRCT variants, including C1697R, R1699W, A1708E, S1715R, G1738E, and M1775R, as well as the truncation mutants, disrupt transactivation function, whereas mutations with no effect on structure (M1652I and R1699Q) are fully active in these assays. However, it has yet to be determined whether BRCT protein misfolding causes BRCA1 tumor suppressor inactivation via BRCA1 transcription function, DNA repair function, or both.

*The Protease-based Assay for Ranking BRCT-destabilizing Effects*—Proteolytic degradation proceeds via an unfolded state for small globular proteins (51, 52) indicating that a correlation between proteolytic resistance and the thermodynamic stability of a protein may exist. This principle forms the basis for phage-based proteolytic selection methods where the evolution of proteins with increased thermodynamic stability closely follows the selection of polypeptides with enhanced resistance to degradation by increasing concentration of protease (53, 54). Thus, the application of a protease-based assay to assess the structural consequences of missense mutations on the BRCT provides a quick, effective, complimentary method to categorize

in triplicate with *error bars* reflecting the standard deviations. *Red*, highly destabilizing mutation (BRCT variant is >60% degraded at 6 μg/ml trypsin; *blue*, intermediately destabilizing mutation (BRCT variant is >60% degraded at 60 μg/ml trypsin); *black/gray*, no destabilizing effect (similar to wild type tryptic sensitivity). *E–H*, structural distribution of BRCA1-BRCT missense mutations. *A* and *E*, surface mutations; *B* and *F*, interface mutations; *C* and *G*, BRCA1 fold mutations; *D* and *H*, BRCT fold mutations.



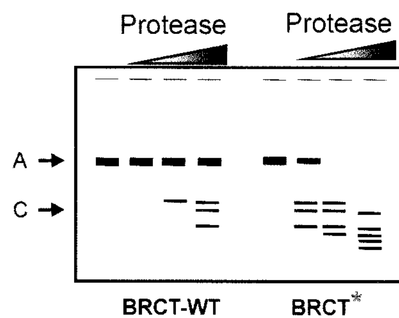




FIG. 6. **Proposed application of BRCT protease susceptibility assays in BRCA1 screening protocols.** *A*, the commonly used protein truncation test could be modified to include a protease digestion step. This method typically generates coding sequence (and protein) from both alleles in an individual. In the case of a heterozygote carrier for missense mutation, highly destabilizing mutants would result in a 50% reduction in protein amounts of the BRCT. The quantitative nature of the protease susceptibility assay would allow one to monitor the disappearance of protein species "*A*." Proteolytic fragments (species "*C*") are also generated in a predictable manner for the wild type protein. Greater sensitivity for the assay could thus be achieved by monitoring the ratio of protein species C/A. For truncating mutations, truncated protein products ("*B*") are produced that would be rapidly degraded by protease. *B*, BRCT missense mutations identified by sequencing could be generated by PCR and transcribed directly from PCR products, eliminating the need for a cloning step.

and rank the extent of destabilization of the mutant BRCT proteins.

A recent biophysical assessment of the effects of 8 missense substitutions and the truncation Y1853ter on the thermodynamic stability of the BRCT revealed that four of these missense mutations and the truncation were highly destabilizing and could not be produced as soluble protein in *Escherichia coli* (14). All four of these missense mutants, A1708E, G1738E, G1788V, and W1837R and the truncation show extreme sensitivity to tryptic digestion (Table I and Figs. 4 and 5). The remaining four (M1775R, M1783T, V1808A, and V1833M) can be produced recombinantly, but destabilize the protein by 3.5–5.5 kcal/mol. Two of these, M1775R and M1783T, show an intermediate sensitivity to proteolysis. Six of the seven other BRCT mutant proteins (V1696L, R1699W, R1751Q, M1783T, V1809F, and Y1853C) with intermediate protease sensitivity are soluble in *E. coli* when expressed at 20 °C (Table I). Altogether, these data indicate a three-tiered hierarchy of destabilizing effects inferred from the proteolytic data is consistent with results obtained from solubility analysis and direct thermodynamic measurements of BRCT protein stability. Highly destabilizing mutations show sensitivity to low levels of trypsin and tend to be degraded or insoluble when expressed in *E. coli*. Intermediate thermodynamically destabilizing mutations are sensitive to moderate levels of protease and can be produced in soluble form in *E. coli*.

The remaining set of mutations may not affect the folding detected by the proteolysis assay and yet still affect the functional properties of human BRCT. The computational methods we have explored represent a first attempt to identify alternative correlates within this class of disease predisposing substitutions. The computational methods were largely consistent with the proteolysis data, whether or not there was an effect on protein stability. The purely sequence-based computational methodology was more consistent with the experimental evidence than the structure- and sequence-based approach. Whether the discrepancy can be interpreted or not remains to be seen through further studies. Methods for predicting the

biological consequences of amino acid substitutions is an area of active research, especially because the genome initiatives are discovering too large a number of amino acid altering genetic variants with potential effects on biological function for experimental analysis (for example, see Refs. 55 and 56).

*Detection of BRCT Mutations*—The observations that the BRCA1 BRCT domains form a proteolytically resistant domain and that cancer-predisposing BRCT variants (but not benign single amino acid substitutions) have compromised stability indicate that a protease-based screen for mutant BRCT conformations could be incorporated into routine BRCA1 screening protocols. The protease-based assay we have used tests the stability of BRCT domains expressed in commercially available reticulocyte lysates. These lysates are employed in protein truncation test genetic screens that have been used for detecting BRCA1 mutations (5, 57, 58). In this method, patient-isolated RNA is reverse-transcribed to generate a cDNA that is then amplified using oligonucleotides that target a coding region of the protein that is to be tested for protein truncating effects. The cDNA message is then transcribed/translated and radiolabeled within the lysate, and the presence of truncated protein product is visualized by SDS-PAGE. Although effective at detecting frameshifts, nonsense and deletion mutations that lead to truncation of the expressed protein message, this technique is incapable of identifying missense substitutions.

An adaptation of the protein truncation test, where a protease digestion step is added could be appropriate for the detection of the large majority of cancer-associated BRCT mutations (Fig. 6*A*). Here, oligonucleotides would be specifically designed to amplify BRCT coding sequence (amino acids 1646–1863) from patient samples, and the translation step would be followed by a trypsinolysis series. This test would have the distinct advantage of sensing the protein destabilizing effects of both missense and truncation mutations. Conservative estimates indicate it could detect as much as 80% of the cancer-associated mutations that fall within the BRCT coding region. Alternatively, for cases where a BRCT missense mutation has already been detected by sequencing, the mutant BRCT coding sequence could be produced by PCR (Fig. 6*B*). Direct transcription/translation from the PCR product, followed by protein digestion, would provide a quick, relatively inexpensive test for mutant BRCT conformations.

## CONCLUSIONS

Greater than 60% of clinically relevant BRCA1 mutations delete a portion of or all of the BRCT domains, and the majority of BRCT missense alterations tested that target the three key classes of BRCT folding determinants (BRCT fold, BRCA1 fold, and interface mutations) are destabilizing. It is apparent that BRCT destabilization or loss of function through truncation or missense substitution is sufficient to confer disease predisposition in carriers for these alleles. Such mutations are comparable to the subset of $\beta$-sandwich and zinc binding mutations that unfold the core DNA binding domain of the p53 tumor suppressor (reviewed in Ref. 22). Conversely, the p53 core domain mutation data base is largely populated by mutations that have little effect on stability, but directly target residues involved in sequence-specific DNA binding. The identification of analogous cancer-associated mutations that are not destabilizing but disrupt specific BRCT protein-protein or protein-DNA binding would provide strong support for the role of these interactions in mediating BRCA1 tumor suppressor function. To this end, two patient-derived mutations (F1695L and V1696L) on the surface of the BRCT that affect BACH1 binding have recently been identified (28). Further biochemical and structural characterization of these interactions will be necessary to confirm the nature of these defects.

We have established a set of probability and protease-based criteria on which we can define the structural effects of mutation on the BRCT at the protein level. The early identification of carriers of potentially deleterious *BRCA1* alleles is an essential component of breast and ovarian cancer screening programs that facilitates detection, surveillance, and prevention of tumor growth. Further development of complimentary methods that test the destabilizing and biological repercussions of missense variants will provide clinicians and researchers with important tools to unravel BRCA1 function and misfunction.

## REFERENCES


1. Nathanson, K. L., Wooster, R., Weber, B. L., and Nathanson, K. N. (2001) *Nat. Med.* **7,** 552–556
2. Monteiro, A. N. (2000) *Trends Biochem. Sci.* **25,** 469–474
3. Scully, R., and Livingston, D. M. (2000) *Nature* **408,** 429–432
4. Venkitaraman, A. R. (2002) *Cell* **108,** 171–182
5. Shattuck-Eidens, D., McClure, M., Simard, J., Labrie, F., Narod, S., Couch, F., Hoskins, K., Weber, B., Castilla, L., Erdos, M., Brody, L., Friedman, L., Ostermeyer, E., Szabo, C., King, M.-C., Jhanwar, S., Offit, K., Norton, L., Gilewski, T., Lubin, M., Osborne, M., Black, D., Boyd, M., Steel, M., Ingle, S., Haile, R., Lindblom, A., Olsson, H., Borg, A., Bishop, D. T., Solomon, E., Radice, P., Spatti, G., Gayther, S., Ponder, B., Warren, W., Stratton, M., Liu, Q., Fujimura, F., Lewis, C., Skolnick, M. H., and Goldgar, D. E. (1995) *JAMA* **273,** 535–541
6. Couch, F. J., and Weber, B. L. (1996) *Hum. Mutat.* **8,** 8–18
7. Shen, D., and Vadgama, J. V. (1999) *Oncol. Res.* **11,** 63–69
8. Sharan, S. K., Wims, M., and Bradley, A. (1995) *Hum. Mol. Genet.* **4,** 2275–2278
9. Chen, K. S., Shepel, L. A., Haag, J. D., Heil, G. M., and Gould, M. N. (1996) *Carcinogenesis* **17,** 1561–1566
10. Joukov, V., Chen, J., Fox, E. A., Green, J. B., and Livingston, D. M. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98,** 12078–12083
11. Friedman, L. S., Ostermeyer, E. A., Szabo, C. I., Dowd, P., Lynch, E. D., Rowell, S. E., and King, M. C. (1994) *Nat. Genet.* **8,** 399–404
12. Miki, Y., Swensen, J., Shattuck-Eidens, D., Futreal, P. A., Harshman, K., Tavtigian, S., Liu, Q., Cochran, C., Bennett, L. M., Ding, W., Bell, R., Rosenthal, J., Hussey, C., Tran, T., McClure, M., Frye, C., Hattier, T., Phelps, R., Haugen-Strano, A., Katcher, H., Yakumo, K., Gholami, Z., Shaffer, D., Stone, S., Bayer, S., Wray, C., Bogden, R., Dayananth, P., Ward, J., Tonin, P., Narod, S., Bristow, P. K., Norris, F. H., Helvering, L., Morrison, P., Rosteck, P., Lai, M., Barrett, J. C., Lewis, C., Neuhausen, S., Cannon-Albright, L., Goldgar, D., Wiseman, R., Kamb, A., and Skolnick, M. H. (1994) *Science* **266,** 66–71
13. Williams, R. S., Green, R., and Glover, J. N. (2001) *Nat. Struct. Biol.* **8,** 838–842
14. Ekblad, C. M., Wilkinson, H. R., Schymkowitz, J. W., Rousseau, F., Freund, S. M., and Itzhaki, L. S. (2002) *J. Mol. Biol.* **320,** 431–442
15. Williams, R. S., and Glover, J. N. (2003) *J. Biol. Chem.* **278,** 2630–2635
16. Scully, R., Ganesan, S., Vlasakova, K., Chen, J., Socolovsky, M., and Livingston, D. M. (1999) *Mol. Cell* **4,** 1093–1099
17. Yarden, R. I., and Brody, L. C. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 4983–4988
18. Cantor, S. B., Bell, D. W., Ganesan, S., Kass, E. M., Drapkin, R., Grossman, S., Wahrer, D. C., Sgroi, D. C., Lane, W. S., Haber, D. A., and Livingston, D. M. (2001) *Cell* **105,** 149–160
19. Yu, X., Wu, L. C., Bowcock, A. M., Aronheim, A., and Baer, R. (1998) *J. Biol. Chem.* **273,** 25388–25392
20. Li, S., Chen, P. L., Subramanian, T., Chinnadurai, G., Tomlinson, G., Osborne, C. K., Sharp, Z. D., and Lee, W. H. (1999) *J. Biol. Chem.* **274,** 11334–11338
21. Ludwig, T., Fisher, P., Ganesan, S., and Efstratiadis, A. (2001) *Genes Dev.* **15,** 1188–1193
22. Bullock, A. N., and Fersht, A. R. (2001) *Nat. Rev. Cancer* **1,** 68–76
23. Futreal, P. A., Liu, Q., Shattuck-Eidens, D., Cochran, C., Harshman, K., Tavtigian, S., Bennett, L. M., Haugen-Strano, A., Swensen, J., and Miki, Y. *et al.* (1994) *Science* **266,** 120–122
24. Hayes, F., Cayanan, C., Barilla, D., and Monteiro, A. N. (2000) *Cancer Res.* **60,** 2411–2418
25. Vallon-Christersson, J., Cayanan, C., Haraldsson, K., Loman, N., Bergthorsson, J. T., Brondum-Nielsen, K., Gerdes, A. M., Moller, P., Kristoffersson, U., Olsson, H., Borg, A., and Monteiro, A. N. (2001) *Hum. Mol. Genet.* **10,** 353–360
26. Carvalho, M. A., Billack, B., Chan, E., Worley, T., Cayanan, C., and Monteiro, A. N. (2002) *Cancer Biol. Ther.* **1,** 502–508
27. Worley, T., Vallon-Christersson, J., Billack, B., Borg, A., and Monteiro, A. N. (2002) *Cancer Biol. Ther.* **1,** 497–501
28. Joo, W. S., Jeffrey, P. D., Cantor, S. B., Finnin, M. S., Livingston, D. M., and Pavletich, N. P. (2002) *Genes Dev.* **16,** 583–593
29. Sodeoka, M., Larson, C. J., Chen, L., Leclair, K. P., and Verdine, G. L. A. (1993) *Bioorg. Med. Chem. Lett* **3,** 1089
30. Horton, R. M., Ho, S. N., Pullen, J. K., Hunt, H. D., Cai, Z., and Pease, L. R. (1993) *Methods Enzymol.* **217,** 270–279
31. Esnouf, R. M. (1997) *J. Mol. Graph Model.* **15,** 132–134
32. Esnouf, R. M. (1999) *Acta Crystallogr. D Biol. Crystallogr.* **55,** 938–940
33. Chasman, D., and Adams, R. M. (2001) *J. Mol. Biol.* **307,** 683–706
34. Markiewicz, P., Kleina, L. G., Cruz, C., Ehret, S., and Miller, J. H. (1994) *J. Mol. Biol.* **240,** 421–433
35. Rennell, D., Bouvier, S. E., Hardy, L. W., and Poteete, A. R. (1991) *J. Mol. Biol.* **222,** 67–88

36. Henikoff, S., Henikoff, J. G., and Pietrokovski, S. (1999) *Bioinformatics* **15,** 471–479
37. Zhang, X., Morera, S., Bates, P. A., Whitehead, P. C., Coffer, A. I., Hainbucher, K., Nash, R. A., Sternberg, M. J., Lindahl, T., and Freemont, P. S. (1998) *EMBO J.* **17,** 6404–6411
38. Huyton, T., Bates, P. A., Zhang, X., Sternberg, M. J., and Freemont, P. S. (2000) *Mutat. Res.* **460,** 319–332
39. Callebaut, I., and Mornon, J. P. (1997) *FEBS Lett.* **400,** 25–30
40. Bork, P., Hofmann, K., Bucher, P., Neuwald, A. F., Altschul, S. F., and Koonin, E. V. (1997) *FASEB J.* **11,** 68–76
41. Deffenbaugh, A. M., Frank, T. S., Hoffman, M., Cannon-Albright, L., and Neuhausen, S. L. (2002) *Genet. Test* **6,** 119–121
42. Monteiro, A. N., August, A., and Hanafusa, H. (1997) *Am. J. Hum. Genet.* **61,** 761–762
43. Sjolander, K., Karplus, K., Brown, M., Hughey, R., Krogh, A., Mian, I. S., and Haussler, D. (1996) *Comput. Appl. Biosci.* **12,** 327–345
44. Chapman, M. S., and Verma, I. M. (1996) *Nature* **382,** 678–679
45. Monteiro, A. N., August, A., and Hanafusa, H. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 13595–13599
46. Haile, D. T., and Parvin, J. D. (1999) *J. Biol. Chem.* **274** 2113–2117
47. Somasundaram, K., Zhang, H., Zeng, Y. X., Houvras, Y., Peng, Y., Zhang, H., Wu, G. S., Licht, J. D., Weber, B. L., and El-Deiry, W. S. (1997) *Nature* **389,** 187–190
48. Harkin, D. P., Bean, J. M., Miklos, D., Song, Y. H., Truong, V. B., Englert, C., Christians, F. C., Ellisen, L. W., Maheswaran, S., Oliner, J. D., and Haber, D. A. (1999) *Cell* **97,** 575–586
49. Scully, R., Anderson, S. F., Chao, D. M., Wei, W., Ye, L., Young, R. A., Livingston, D. M., and Parvin, J. D. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94,** 5605–5610
50. Anderson, S. F., Schlegel, B. P., Nakajima, T., Wolpin, E. S., and Parvin, J. D. (1998) *Nat. Genet.* **19,** 254–256
51. Imoto, T., Yamada, H., and Ueda, T. (1986) *J. Mol. Biol.* **190,** 647–649
52. Wang, L., and Kallenbach, N. R. (1998) *Protein Sci.* **7,** 2460–2464
53. Sieber, V., Pluckthun, A., and Schmid, F. X. (1998) *Nat. Biotechnol.* **16,** 955–960
54. Pedersen, J. S., Otzen, D. E., and Kristensen, P. (2002) *J. Mol. Biol.* **323,** 115–123
55. Ramensky, V., Bork, P., and Sunyaev, S. (2002) *Nucleic Acids Res.* **30,** 3894–3900
56. Saunders, C. T., and Baker, D. (2002) *J. Mol. Biol.* **322,** 891–901
57. Petrij-Bosch, A., Peelen, T., van Vliet, M., van Eijk, R., Olmer, R., Drusedau, M., Hogervorst, F. B., Hageman, S., Arts, P. J., Ligtenberg, M. J., Meijers-Heijboer, H., Klijn, J. G., Vasen, H. F., Cornelisse, C. J., van't Veer, L. J., Bakker, E., van Ommen, G. J., and Devilee, P. (1997) *Nat. Genet.* **17,** 341–345
58. Vossen, R., and den-Dunnen, J. T. (1999) *Current Protocols in Human Genetics*, John Wiley & Sons, Inc, New York, pp. 9.11.1–9.11.21